A STUDY ON GLYCOSYLATION PATTERN OF SERUM TRANSFERRIN IN PATIENTS WITH CLINICALLY SUSPECTED CONGENITAL GLYCOSYLATION DISORDERS

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BONAFIDE CERTIFICATE

This to certify that this dissertation work entitled **"A STUDY ON GLYCOSYLATION PATTERN OF SERUM TRANSFERRIN IN PATIENTS WITH CLINICALLY SUSPECTED CONGENITAL GLYCOSYLATION DISORDERS"** is the original bonafide work done by **Dr.A.PREETHI**, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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DECLARATION

I, **Dr.A. PREETHI,** Post Graduate, Institute of Biochemistry, Madras Medical College, solemnly declare that the dissertation titled **"A STUDY ON GLYCOSYLATION PATTERN OF SERUM TRANSFERRIN IN PATIENTS WITH CLINICALLY SUSPECTED CONGENITAL GLYCOSYLATION DISORDERS"** is the bonafide work done by me at Institute of Biochemistry, Madras Medical College under the expert guidance and supervision of **Prof.Dr.K.RAMADEVI,** M.D., Professor and Head, Institute of Biochemistry, Madras Medical College. The dissertation is submitted to the Tamil Nadu Dr.M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch XIII) in Biochemistry.

Place: Chennai

Date: **Dr.A.PREETHI**

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CONTENTS

ABBREVIATIONS

1. ER	: ENDOPLASMIC RETICULUM
2. UDP	: URIDINE DIPHOSPHATE
3. GDP	: GUANOSINE DIPHOSPHATE
4. CMP	: CYTIDINE MONOPHOSPHATE
5. NeuAc	: NEURAMINIC ACID
6. Gal	: GALACTOSE
7. UDP-Glc	: URIDINE DIPHOSPHATE GLUCOSE
8. UDP-Gal	: URIDINE DIPHOSPHATE GALACTOSE
9. GDP-Man	: GUANOSINE DIPHOSPHATE MANNOSE
10. GDP-Fuc	: GUANOSINE DIPHOSPHATE FUCOSE
11. UDP-GalNAc	: URIDINE DIPHOSPHATE -N- CETYLGALACTOSAMINE
12. UDP-GlcNAc	: URIDINE DIPHOSPHATE UDP-N-ACETYLGLUCOSAMINE
13. UDP-Xyl	: URIDINE DIPHOSPHATE XYLOSE
14. NSTs	: NUCLEOTIDE SPECIFIC SUGAR TRANSPORTERS
15. Asn	: ASPARAGINE
16. RIA	: RADIO IMMUNOASSAY
17. GlcNAc	: N-ACETYLGLUCOSAMINE
18. Dol-P-Man	: DOLICHOL-PHOSPHATE-MANNOSE
19. Dol-P-Gluc	: DOLICHOL-PHOSPHATE-GLUCOSE
20. GPI	: GLYCOSYLPHOSPHATIDYLINOSITOL
21. DAF	: DECAY ACCELERATING FACTOR
22. ICAM1	: INTERCELLULAR ADHESION MOLECULE-1
23. PECAM	: PLATELET ENDOTHELIAL CELL ADHESION MOLECULE-1
24. MHC	: MAJOR HISTOCOMPATIBILITY COMPLEX
25. ATP	: ADENOSINE TRIPHOSPHATE
26. MS	: MASS SPECTROMETRY

27. ZP-1 : ZONA PELLUCID GLYCOPROTEIN 1

28. ZP-2 : ZONA PELLUCIDA GLYCOPROTEIN 2

29. ZP-3 : ZONA PELLUCIDA GLYCOPROTEIN 3

30. PM : PLASMA MEMBRANE

31. EPO : ERYTHROPOIETIN

32. NMR spectroscopy : NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

33. Cdna : COMPLEMENTARY DNA

34. LAD II : LEUKOCYTE ADHESION DEFICIENCY 2

35. CDG : CONGENITAL DISORDERS OF GLYCOSYLATION

36. IEF : ISOELECTRIC FOCUSSING

37. PAGE-IEF : POLYACRYLAMIDE GEL ISOELECTRIC FOCUSSING

38. α-DG : ALPHA DYSTROGLYCAN

39. Man-6-P : MANNOSE -6-PHOSPHATE

40.IGF-II : INSULIN-LIKE GROWTH FACTOR II.

41.MEKC : MICELLAR ELECTROKINETIC CHROMATOGRAPHY

42. PIG-A : PHOSPHATIDYL INOSITOL GLYCAN CLASS A

43. LLO : LIPID LINKED OLIGOSACCHARIDE

44.PMM2 : PHOSPHOMANNOMUTASE 2

45. MPI : PHOSPHOMANNOSE ISOMERASE

46. CDT : CARBOHYDRATE DEFICIENT TRANSFERRIN

47. OLP : OPTICAL LIGHT PATH

48. CZE : CAPILLARY ZONE ELECTROPHORESIS

49. EDTA : ETHYLENEDIAMINETETRAACETIC ACID

50. CE : CAPILLARY ELECTROPHORESIS

Introduction

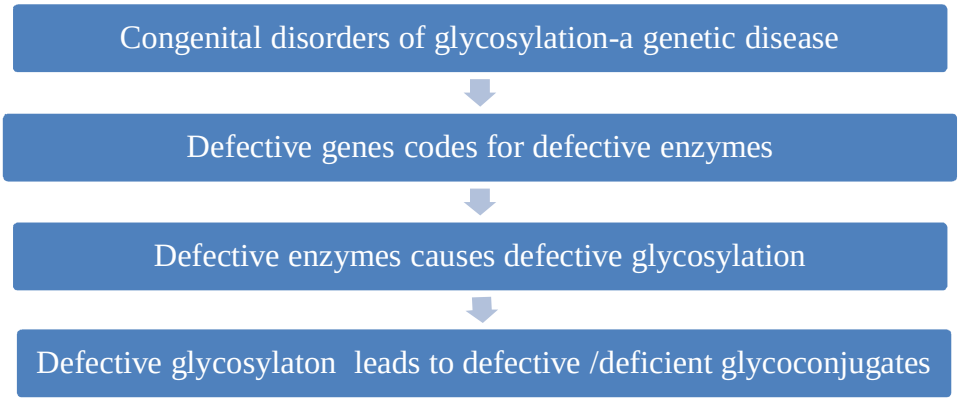
INTRODUCTION

Glycosylation is the process of adding sugar moieties to the proteins or lipids enzymatically in order to produce Glycoconjugates such as Glycoproteins and Glycolipids.

Glycoproteins and Glycolipids have multiple significant role in cell biology[1] such as Protein folding, Protein stability, Transport of proteins, Cell to cell recognition, Cell signaling, Cell membrane integrity and Host defense

Congenital disorders of glycosylation refers to a group of metabolic disorders which is due to either deficient or defective glycosylation of glycoproteins or glycolipids in the tissue of the patient [2].

Earlier this disorder was termed as carbohydrate deficient glycoprotein syndrome.



Congenital Disorders of Glycosylation (CDG) is a genetic disorder transmitted in an Autosomal recessive pattern .The clinical presentation of CDG is a broad spectrum from nearly normal condition with mild clinical symptoms to serious lethal malfunction of different organ systems.The nervous system,

intestines and muscles are the most affected systems in this disorder. This group of disorder is also known as CDG syndromes. Though CDG has a wide variety of clinical presentation, neurological impairment is the predominant symptom in many subtypes of CDG [3].

The first case of CDG was reported by Dr. Jaeken and his colleagues in 1980. Later in the subsequent years about 100 different types of CDG have been identified [3].

The broad clinical presentation of the CDG challenges physicians in diagnosing this disorder. One way of diagnosing this defect is by checking the glycosylation pattern of a glycoprotein. Defective glycosylation pattern of the glycoprotein gives a clue for the diagnosis of CDG. Transferrin being the widely abundant glycoprotein in the serum can be checked for its glycosylation pattern. Glycosylation pattern of transferrin was studied by isoelectric focussing technique which is the established gold standard technique for the diagnosis of CDG. The technique of isoelectric focussing is labour intensive and time consuming. Since fast and accurate diagnosis of metabolic disease is very essential to provide the best care for the patients, need was felt for other automated & rapid techniques to study the glycosylation pattern. This lead to the evolution of new techniques[4] such as

a. Capillary zone electrophoresis,

b. Micro-column separation combined with turbidimetry,

c. High-performance liquid chromatography,

d. Radioimmunoassay (RIA)

e. Enzyme immune assay (EIA)

f. Electro spray mass spectrometry.

Among the automated techniques for diagnosing congenital disorders of glycosylation capillary zone electrophoresis is simple and less time consuming technique and can be conveniently used for screening large number of samples [4]. Diagnosis of CDG should then be confirmed by the isoelectric focussing of serum transferrin, enzyme analysis and genetic studies.

Although at present there is no specific treatment that cures CDG, symptomatic treatment reduces the morbidity in these patients. Treatment of CDG requires a panel of specialists. The panel should include paediatrician, neurologist, ophthalmologist, surgeons, gastroenterologist, cardiologist, speech pathologist, laboratory professionals and other health care professionals who have to systematically plan and act for the affected patient's treatment. Three types of CDG have an existing therapy. Researchers are studying enzyme replacement therapy and gene therapy to cure this disorder.

Therefore screening the patients with symptoms of CDG and delineating them from other metabolic disorders is essential for future therapy.

Review of Literature

REVIEW OF LITERATURE

GLYCOBIOLOGY

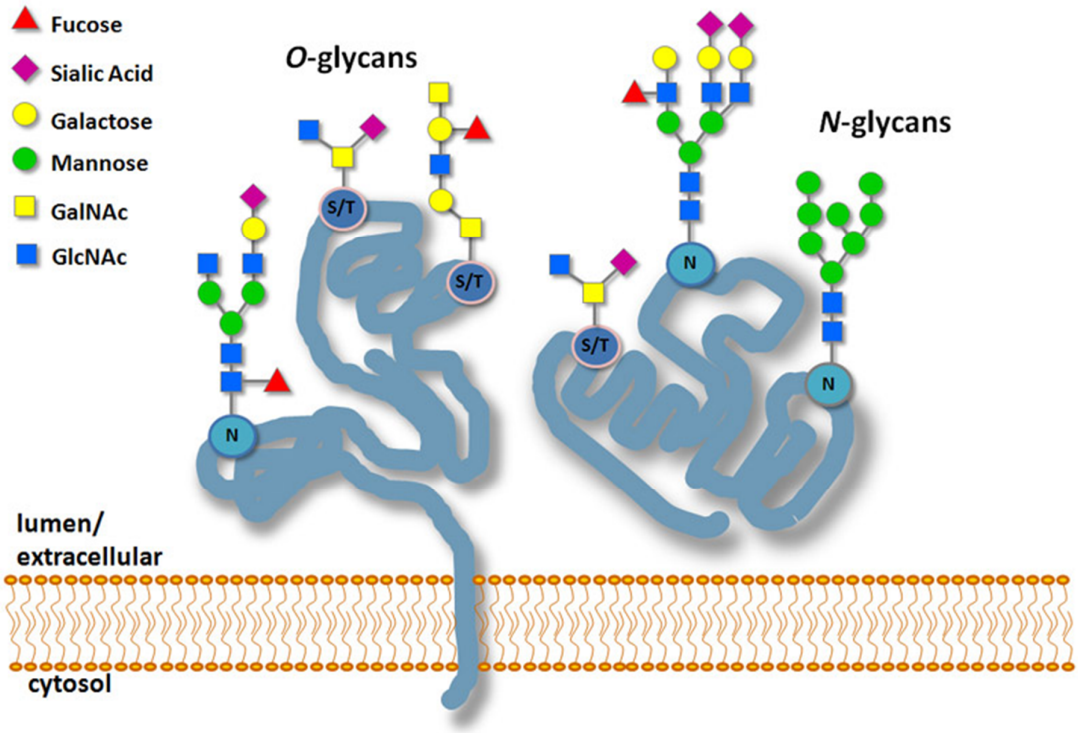
"Glycobiology is defined as the study of the role of sugars in health and disease" [5]. "The glycome is defined as the entire complement of sugars, whether free or present in more complex molecules, of an organism.

"Glycomics is the comprehensive study of glycomes, including genetic, physiologic, pathologic and other aspects" [5].

Certain oligosaccharide molecules contain specific biologic information, depending upon their constituent sugars, their linkages and their sequences. The biologic information is expressed via the interactions between specific sugars in glycoconjugates with the proteins or other molecules. Which lead to changes of cellular activity. Thus, the so called "sugar code of life" (one of the principal aims of glycomics) requires the description of the interactions that sugars and sugar containing molecules participate in, and the results of these interactions on cellular behavior [6].

Research in glycomics will provide the structural information on glycoconjugates, disclose "the sugar code of life". It may also expose many significant sugar dependent biologic interactions that are vital for life. This can also endow with targets for drugs and other therapies. Since appropriate analytical technical are now available, the pace of research in glycomics is accelerating.

Figure 1: *O*-linked glycans and *N*-linked glycans



(Courtesy: untitled illustration of O-linked and N-linked glycans,Retrieved on september 1,2016 from http://www.neb.com/applications/glycobiology)

GLYCOSYLATION

Glycosylation refers to the process of attaching the carbohydrate chains to proteins, lipids, or other organic molecules. Glycosylation is an enzyme mediated site-specific process, while glycation is not enzyme mediated.

"Glycation is a non enzymatic process of adding sugar moiety to a non carbohydrate molecule"[1]. The enzymatic process of glycosylation produces glycoconjugates that is of vast clinical significance. "Glycoconjugate is defined as the molecule containing one or more carbohydrate chains covalently linked to protein or lipid"[1].Glycoproteins are one class of glycoconjugates.Most of the proteins undergo glycosylation reaction after being synthesized in the rough endoplasmic reticulum. As S Hiroyuki Kajirightly states that"It has been estimated that approximately 50 % of eukaryotic proteins have sugars attached, so that glycosylation is the most frequent post translational modification of proteins"[7]. Almost all the plasma proteins of humans with the notable exception of albumin are glycoproteins. Glycoproteins are present in almost all organisms i.e. from viruses and bacteria to human beings,The carbohydrate content of glycoprotein ranges from 1% to over 85% by weight [8].

Sugar donors involved in glycosylation:

In majority of the biosynthetic reactions only the nucleotide sugars are involved in glycosylation. Neither the free sugars nor the phosphorylated sugars are involved in glycosylation reactions. Uridine diphosphate glucose (UDP-Glc) was the first nucleotide sugar reported to be involved in glycosylation reactions

.Later other nucleotde sugars were found to be involved in glycosylation reactions. Some of the common nucleotide sugars involved in the synthesis of glycoproteins are UDP-Gal, UDP-Glc, GDP-Man CMP-NeuAc, GDP-Fuc, UDP-GalNAc, UDP-GlcNAc UDP-Xyl.

It is not clear that why some nucleotide sugars contain UDP and others contain GDP/CMP.The bond or linkage formed between the sugar and the phosphate group in a nucleotide sugar is of high group transfer potential and high energy type. Nucleotide sugars are the active forms of sugars which can be then transferred in the presence of appropriate transferase enzymes to a suitable acceptor.

Transport of sugar donors to the site of glycosylation:

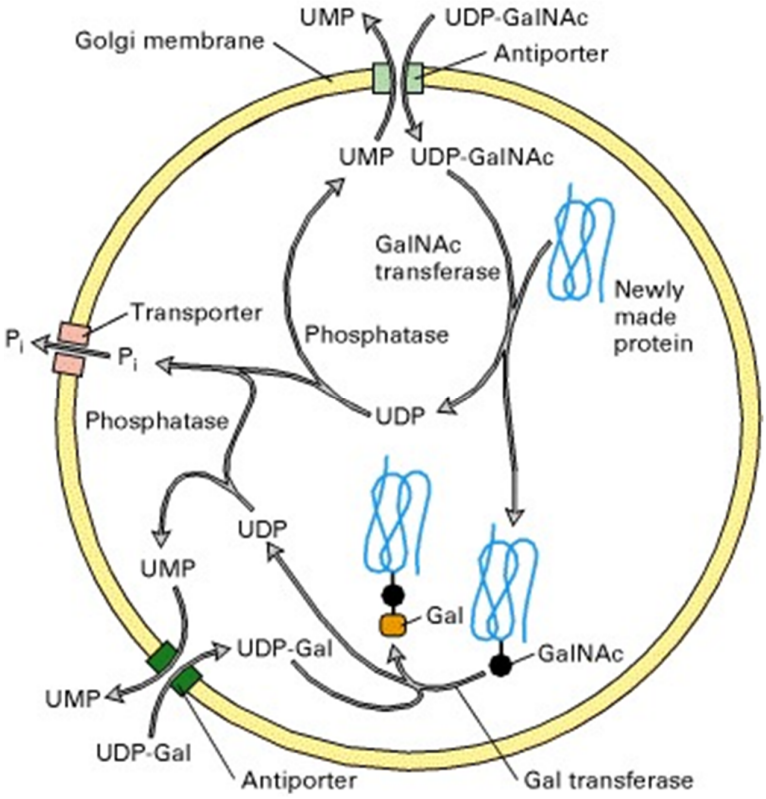
Nucleoside triphosphates are the precursor molecules required for the generation of activated nucleotide sugars. This reaction takes place in the cytosol of cells except the CMP-sialic acids which are formed inside the nucleus.Since most of the glycosylation reactions takes place within the lumen of the golgi complex and/or endoplasmic reticulum,a carrier system is required to transport the activated nucleotide sugars from the cytosol to the lumen of the golgi apparatus across the golgi membrane .These carrier systems are mostly antiport systems i.e.,movement of one molecule of the activated nucleotide sugar is balanced by the transfer of another corresponding nucleotide.This carrier system/transport mechanism thus ensures sufficient concentration of each nucleotide sugar inside the lumen of the golgi apparatus.

The sugar moieties are transported to the endoplasmic reticulum or Golgi apparatus for glycosylation by two mechanisms

1) By dolichol phosphate transporter (dolichol is a polyisoprenoid, which is a metabolic intermediate in cholesterol synthesis)
2) By specific nucleotide sugar transporters (NSTs).

- UDP present in the lumen of the golgi complex is converted to UMP by the action of phosphatase enzyme .
- UMP in the lumen of golgi complex is exchanged for Both UDP-galactose (UDP-Gal) and UDP–*N*-acetylgalactosamine (UDP-GalNAc) into the cytosol. Therefore UDP-Gal and UDP-N-acetylgalactosamine is transferred inside the lumen of the golgi complex.
- This transport mechanism takes place through the two different types of antiporters found in the golgi membrane.
- This transport mechanism is mediate by two enzymes namely galactosyltransferase and *N*-acetylgalactosaminyltransferase .
- A different transporter is found to transfer the inorganic phosphate (P_i) formed during the conversion of UDP to UMP to move out of the the Golgi cistern.
- Other known antiporters permit CMP–*N*-acetylneuraminic acid to enter the lumen of Golgi complex in exchange for CMP.

Figure 2: The transport mechanism for the uptake of nucleotide sugars into Golgi apparatus



(Courtesy: untitled illustration of golgi complex transporters, Retrieved on September 10,2016 from http://www.cryst.bbk.ac.uk/pps97/assignments/projects/emilia/Biosynthesis.HTM)

Glycoconjugates –the end product of glycosylation:

"Glycoconjugates are carbohydrates that are covalently linked to other biological molecules (aglycone) such as amino acids, proteins, lipids and other small molecules"[5].

Classification of glycoconjugates:

The glycoconjugates are classified based upon the type of aglycone molecule attached and on the relative proportions of the carbohydrate/ aglycone parts.

Thus glycoconjugates are classified as [6]

1. Glycoproteins
2. Glycolipids
3. Peptidoglycans
4. Proteoglycans
5. Lipoglycans

Glycoproteins [7]:

Oligosaccharide side chains are covalently attached to polypeptide side chains to form glycoproteins. This covalent attachment happens either as a cotranslational or posttranslational modification of a protein.

The principal oligosaccharide side chains found in glycoproteins are β-D-Glucose, β-D-mannose, α-L-fucose, β-D-Galactose N acetyl galactosamine, N-Acetyl glucosamine, N-Acetyl Neuraminic acid ,xylose.

Bonds involved in a glycoprotein:

There are two types of bonds involved in a glycoprotein:

- Glycosidic bonds between the carbohydrate residues in a glycan molecule.
- The bond between the carbohydrate side chain and the protein molecule.

In a glycan molecule the carbohydrate residues are linked by the glycosidic bonds formed between the carbon 1 and carbon 4 of the glycans. Since the glycosidic bond formation is an energy requiring mechanism, the energy is supplied by the hydrolysis of ATP to ADP [9].while, the addition of a glycan molecule to the protein necessitates the recognition of a consensus sequence."Consensus sequence is defined as a sequence of DNA that has similar structure and function in different organisms" [9].

Three classes of glycans/glycoproteins produced are:

1. *N*-linked glycoproteins
2. *O*-linked glycoproteins
3. Glypiation products.

***N*-linked glycans**: [10]

N-linked glycosylation, is the process of attaching the oligosaccharide to the asparagine aminoacid of the protein molecule through the amide linkage. *N*-linked glycoproteins are the major class of glycoproteins that has been much studied. It includes both membrane bound and circulating glycoproteins.

The synthesis of *N*-linked glycoproteins involve 3 major steps .They are as follows: [10]

1. Dolichol-linked precursor oligosaccharide synthesis
2. Transfer of the precursor oligosaccharide to the protein molecule
3. Processing of the protein linked oligosaccharide

Site of *N*-linked glycosylation:

1. First two steps i.e.dolichol-linked precursor oligosaccharide synthesis, transfer of the precursor oligosaccharide to the protein molecule and initial processing of the oligosaccharide linked protein molecule, takes place in the endoplasmic reticulum (ER).

2. Consecutive processing and modification of the oligosaccharide linked protein molecule takes place in the Golgi complex.

   From the above it is clear that the synthesis of a glycoprotein requires different subcellular organelles i.e. endoplasmic reticulum and Golgi complex. Thus for *N*-linked glycoprotein to get synthesized, it requires an accessibility to different enzymes present within different subcellular organelles. Interestingly all *N*-linked glycoproteins are synthesized from a general core glycan structure. This takes place through a common pathway [10] .The core glycan structure consists of three mannose residues and two *N*-acetyl glucosamine. Following which further modifications of the general core glycan structure into variety of different glycoproteins takes place. [10]

1.Dolichol-linked precursor oligosaccharide synthesis

The first process in the synthesis of dolichol linked precursor oligosaccharide is the formation of dolichol-linked GlcNAc sugar. Dolichol which is an intermediate in the cholesterol synthetic pathway is a lipid molecule made up of repeated isoprenoid units and is attached to the membrane of the ER. Pyrophosphate linkage is formed between the dolichol and the sugar molecules [10].The oligosaccharide chain is then extended by adding together a variety of sugar molecules to the precursor oligosaccharide in a step-wise fashion.

The formation of this precursor oligosaccharide takes place in two phases: [10].

Phase I –it occurs in the cytoplasmic side of ER

Phase II – it occurs in the luminal side of ER.

The resultant precursor molecule which has to be transferred to a protein comprises of 2 GlcNAc, 3 glucose and 9 mannose molecules.

Phase I:

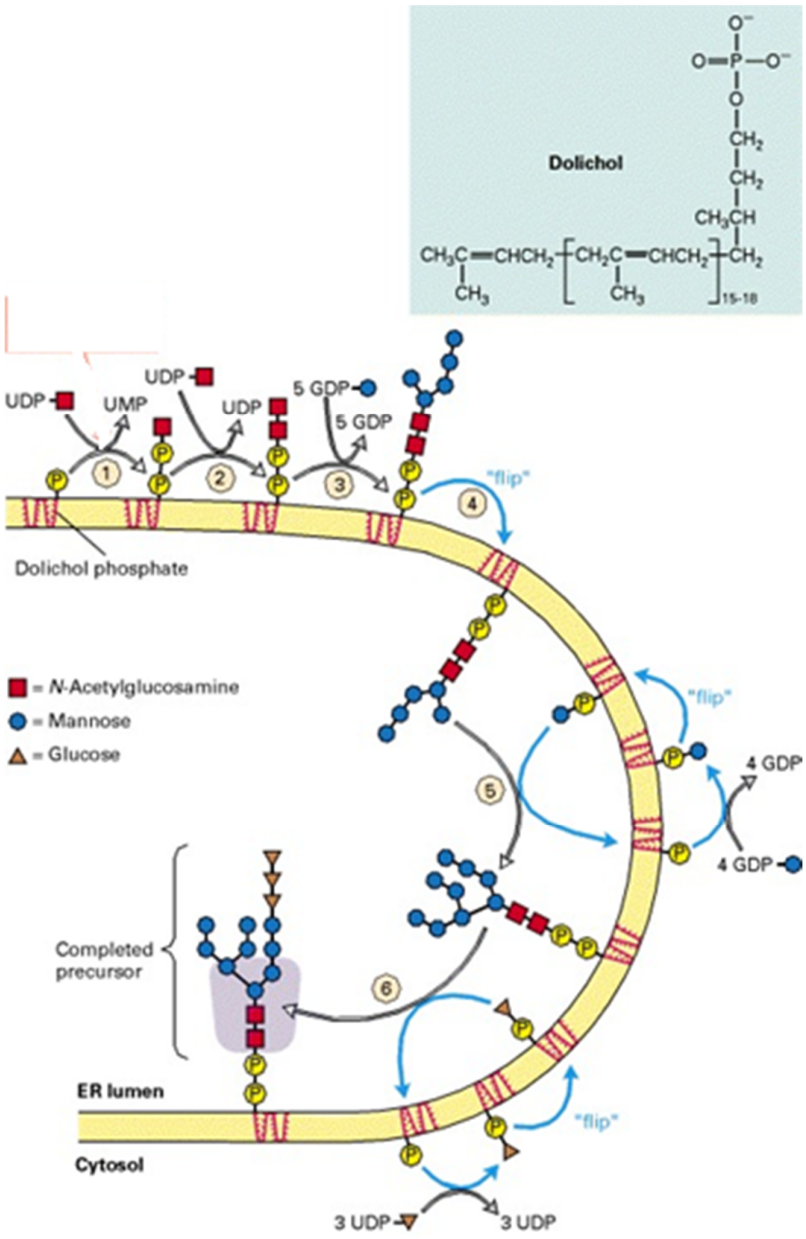
- On the cytoplasmic side of the ER, two UDP-GlcNAc residues are attached to the dolichol molecule.A pyrophosphate linkage is formed between the dolichol and UDP-GlcNAc .
- Glycosyltransferase catalyses the attachment of five GDP-Man residues to the GlcNAc disaccharide
- Thus the resulting precursor molecule in phase one is Dolichol - $GlcNAc_2$ - Man_5

Dolichol - $GlcNAc_2$ - Man_5 is then translocated across the membrane of endoplasmic reticulum to the lumen of the endoplasmic reticulum so that the precursor molecule is made accessible to the enzymes present in the lumen of the endoplasmic reticulum. Enzyme flippase is responsible for this translocation process.

Phase 2:

- In the endoplasmic reticulum, 4 mannose and 3 glucose molecules are attached to the Dolichol - $GlcNAc_2$ - Man_5 present on the luminal side.
- Dol-P-Man & Dol-P-Glc are attached to the dolichol molecule formed in phase one which is then transported from the cytoplasm of the ER into the lumen of ER by the enzyme flippase.
- Thus the resulting precursor molecule in phase two is Dolichol - $GlcNAc_2 – Man_9\text{-}Glc_3$.

Figure 3: Synthesis of precursor oligosaccharide



(Courtesy: untitled illustration of Precursor oligosaccharide synthesis, retrieved on august 4,2016.from http://www.studyblue.com/notes/note/n/lecture-5/deck/5362534)

Dolichol phosphate is strongly hydrophobic and long enough to span a phospholipid bilayer membrane four or five times.

The figure illustrates that:

Steps1 to 3: Two *N*-acetylglucosamine, one phosphate, and five mannose residues from UDP sugars are added one at a time to a dolichol phosphate on the cytosolic face of the ER membrane.

Step 4: Then the dolichol pyrophosphoryl oligosaccharide is flipped to the luminal face

Steps 5, 6: The remaining four mannose and all three glucose residues are added, yielding the completed $Glc_3Man_9(GlcNAc)_2$ precursor, which is transferred to proteins.

In the latter reactions the mannose or glucose is first transferred from a nucleotide sugar to a carrier dolichol phosphate on the cytosolic face of the ER; the carrier is then flipped to the luminal face where the glucose or mannose is transferred to the growing oligosaccharide, and the carrier is flipped back again to the cytosolic face. Five residues in the final precursor are conserved in the structures of all *N*-linked oligosaccharides on secreted proteins

2. Transfer of the precursor oligosaccharide to the protein molecule

After the formation of the precursor oligosaccharide Dolichol - $GlcNAc_2$ – Man_9-Glc_3, it is conveyed to the nascent polypeptide. This process takes place in

the lumen of the ER. Energy required for this reaction is obtained from the cleavage of the pyrophosphate bond between the dolichol glycan molecules.

Before a glycan is relocated to an emerging polypeptide it has to satisfy the following conditions:[10]

1. The primary structure should have asparagine situated in a specific consensus sequence.
2. Asparagine should have to be place precisely in the 3D structure of the protein.i.e. asparagine should be present on the surface of the protein molecule to which the sugar molecules need to be attached.Since sugar are polar molecules, it should not be buried in the interior of the protein.
3. For the initiation of *N*-Linked glycosylation, Asparagine has to present in the luminal side of the endoplasmic reticulum.

The enzyme Oligosaccharyltransferase recognises the consensus sequence and transfer the glycan precursor to the polypeptide molecule in the lumen of the endoplasmic reticulum. *N*-linked glycosylation occurs cotranslationally.

3. Processing of the protein linked oligosaccharide

Processingof *N*-glycan takes place in the endoplasmic reticulum and in the golgi body.

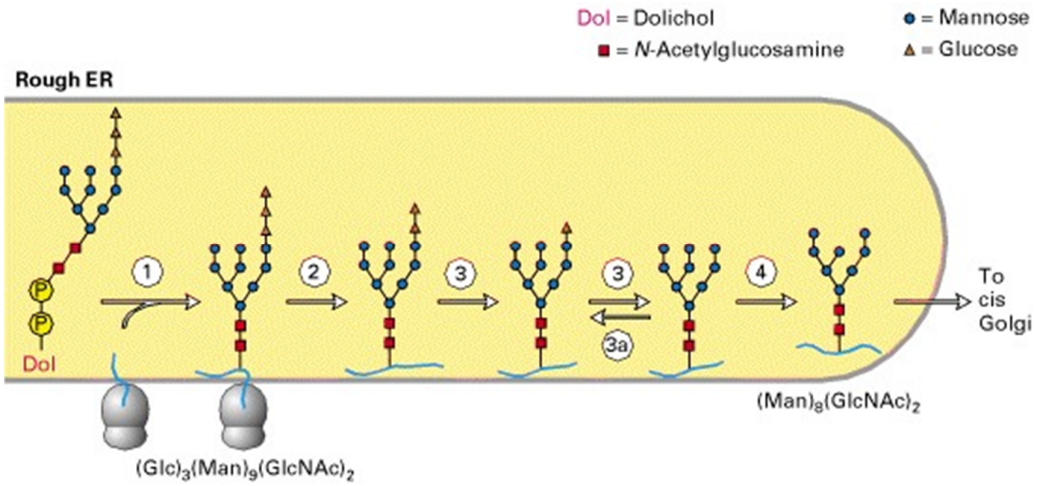
1. Initial processing of the precursor molecule in endoplasmic reticulum
2. Subsequent processing in golgi body

Initial processing of the precursor molecule in endoplasmic reticulum:

It is a quality control step that checks protein folding.If the protein is correctly folded,an enzyme known as glycosidases (glucosidase1 & 2)will remove three glucose moieties from the glycoprotein structure . Glycosidase cleave the glycosidic bonds by using a molecule of water.Once the third glucose moiety is removed from the glycoprotein structure, it moves from ER to the cis golgi . But if the protein is not properly folded (i.e. unfolded or partially folded), a chaperone protein will bind to the misfolded protein to assist in protein folding. The chaperone proteins are calnexin /calreticulin.

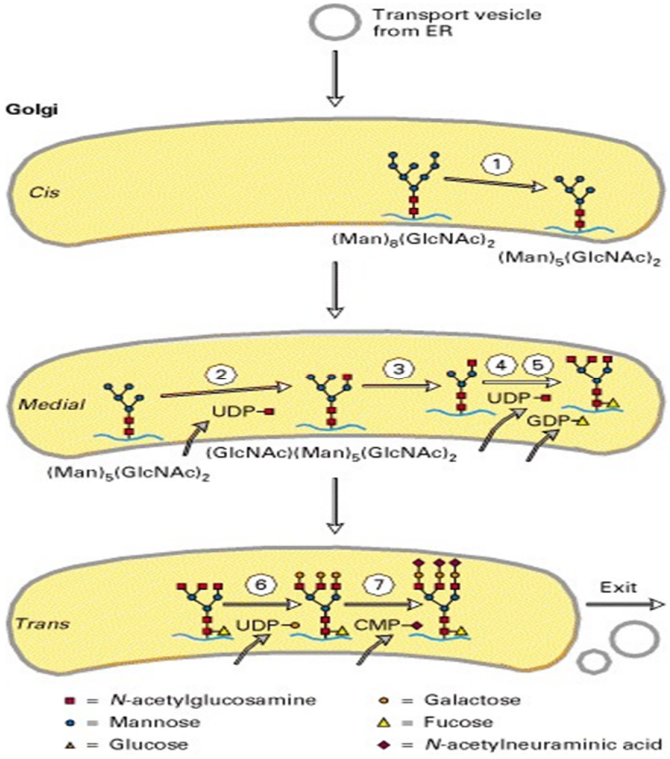
Once these reactions are completed, the newly synthesized protein is then transferred to the golgi for subsequent processing.

Figure 4: Transfer of the precursor oligosaccharide to the protein molecule and initial processing of *N*-linked oligosaccharides (in the rough ER)



(Courtesy: untitled illustration of precursor oligosaccharide transfer, Retrieved on august 10, 2016 fom http//www.studyblue.com/notes/note/n/lecture-5/deck/5362534)

Figure 5: Subsequent processing of precursor oligosaccharide in the golgi body:



(Courtesy: untitled illustration of precursor oligosaccharide processing, retrieved on august 10,2016. from http://www2.nsysu.edu.tw/wzhlab/cell%20biology%20PDF/Ch%2014%20part%20I)

In the cis Golgi three mannose residues are removed. The protein then moves from cis Golgi to the the medial-Golgi. Where, one GlcNAc residue is added and two more mannose residues are removed.folloeing which Two GlcNAc residues are added and One fucose molecule is attached. Then Three galactose molecules are added in the trans golgi and the processing is thus completed. Finally to each galactose residue N-acetylneuraminic acid is added.

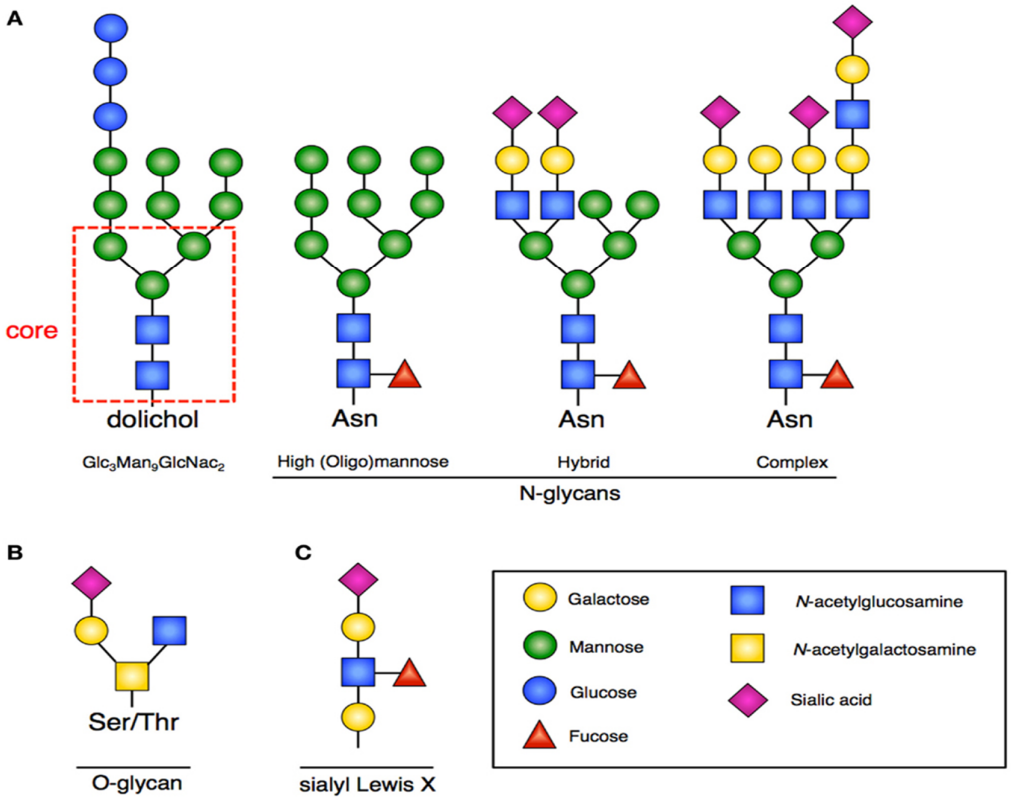
Addition of sugar residues is catalyzed by glycosyltransferases and removal of sugar residues is by glycosidases.

Few mannose residues are detached by enzyme mannosidases in the cis golgi.

In the medial portion of golgi complex- some sugar residues are added by glycosyltransferases which results in the formation of mainly three types of glycoproteins. They are named as high mannose glycans, hybrid glycans and complex glycans depending upon their mannose content.

- High mannose glycans- two *N*-acetylglucosamines with many mannose residues.
- Hybrid glycans-one side of the branch consists of a mannose residues, whereas Nacetylglucosamine recruits a complex branch on the other side.
- Complex glycans- it consists of greater than two *N*-acetylglucosamines along with any number of other kinds of sugar moeities

Figure 6: Type of *N*-glycans



(Courtesy: untitled illustration of N-glycans,retrieved on september 4 ,2016. from http://www.dx.doi.org/10.3389/fped.2015.00054)

_O_-linked glycans:

"_O_-linked glycosylation is defined as the process which involves the attachment of a sugar molecule to the oxygen atom of an aminoacid in a protein" [11]. It occurs in the golgi apparatus .[12]

In other words _O_-linked glycosylation is the process of adding Nacetylgalactosamine to serine /threonine residues in a protein by the enzyme UDP-Nacetylgalactosamine: polypeptide Nacetylgalactosaminyltransferase.

Glycoprotein in humans consists of four types of _O_-glycosidic linkages.

1. GalNAcSer (Thr) bond is the predominant linkage.Usually a Galactose or Neuraminic acid residue is added to the GalNAc, However the composition of the sugars and length of the oligosaccharide varies in the glycoprotein. One example for this type of linkage is mucins.
2. Gal-Gal-Xyl-Ser trisaccharide linkage is the second type of linkage.eg: proteoglycans consists of this type of linkage.
3. Gal-Hydroxylysine (Hyl) linkage is the third type of linkage.eg: collagens contains such linkage.
4. Many nuclear proteins and cytosolic proteins are made up of side chains comprising of a single GlcNAc added to a serine / threonine residue.(GlcNAc-Ser[Thr]).This linkage comprises the fourth type .

Sugars involved in O- linked glycosylation are:

_O_-Nacetylgalactosamine (_O_-GalNAc), _O_-fucose, _O_-glucose, _O-N_acetylglucosamine(_O_-GlcNAc),_O_-mannose

Examples of *O*-linked glycoproteins:

1. Collagen
2. Glycogenin.

In collagen lysine residues are hydroxylated to hydroxylysine to which a galactose residue is added by glycosylation. A molecule of glucose is then added and the structure is elongated . Glycosylation of hydroxylysine take place in the ER and Golgi apparatus [13].

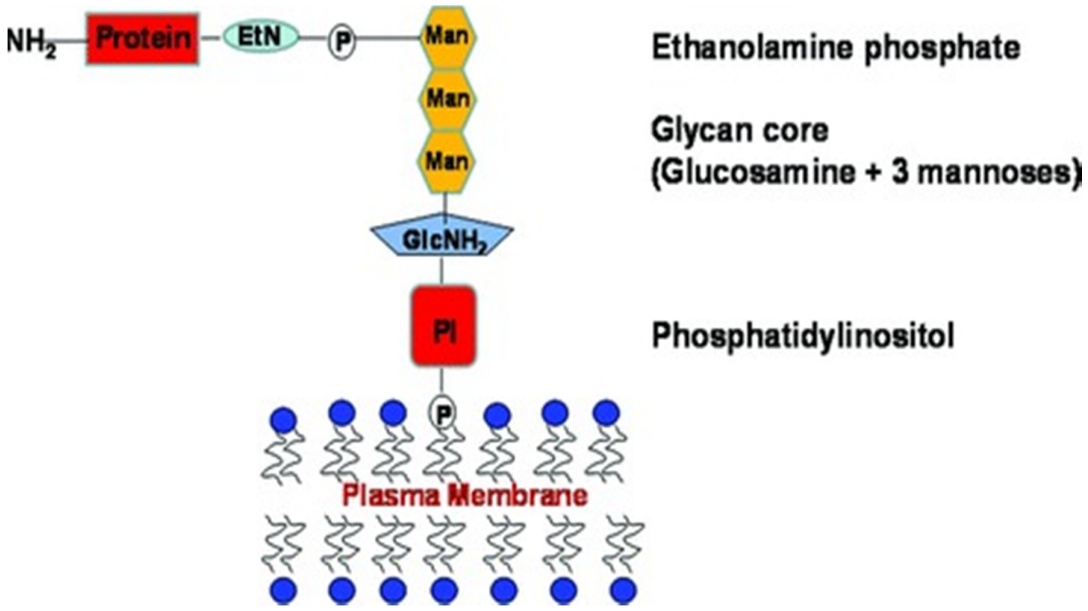
This glycosylation is obligatory for appropriate functioning of the collagen. The only glycosylated tyrosine in nature is the glycogenin. Glycogenin is a glycosyl transferase enzyme required for the polymerization of glucose molecules into glycogen.

Glipiation:

Many membrane associated proteins neither belong to the peripheral nor to transmembrane class. A glycosyl phosphotidyl inositol linkage (GPI) to the carboxy terminal of protein links the protein to the outer aspect of plasma membrane. This type of membrane attachment is referred to as GPI linkage.

The phosphotidyl iositol is linked to the glycan chain. In turn the oligosaccharide is attached through phosphoryl ethanolamine to the carboxy terminal of the amino acid.

Figure 7: GPI anchor



(Courtesy: untitled illustration of GPI anchor,Retrieved on september 4, from http://www.researchgate.net)

It involves atleast 10 reaction coded by 20 different genes.

Examples of GPI linked proteins are :

II. Acetylcholinesterse (red cell membrane)

III. Thy-1-antigen (brain,T-Lymphocytes)

IV. decay accelerating factor (red cell membranes)

V. alkaline phosphatase (intestine,placenta)

VI. 5'nucleotidase (T Lymphocytes)

Hepatocyte mediated removal of certain glycoproteins from plasma

- In 1970s Ashwell and his colleagues performed an experiment to understand the functional significance of oligosaccharide side chains in glycoproteins.
- Rabbit's ceruloplasmin was treated with neuraminidase in vitro.
- Normally the terminal neuraminic acid residues cover the subterminal galactose residues. By treatment with neuraminidase the galactose residue is exposed in the ceruloplasmin.
- When the clearance of an untreated protein is compared with the neuraminidase treated protein,it was found that the neuraminidase treated ceruloplasmin disappears quickly from the circulation
- However, if the neuraminidase treated protein was subjected to treatment with galactosidase, the clearance rate in the plasma was found to be normal.
- Later studies showed that hepatocytes consists of mammalian asialoglycoprotein receptor which identifies the galactose of desialylated

glycoproteins .This process results in endocytosis of the desialylated glycoprotein.

- This work indicated that Gal play an important role in governing the time of residence of certain glycoproteins in the circulation.

Functions of the oligosaccharide chains of glycoproteins:

1. It is involved in the modulation of physicochemical properties of the glycoprotein for ,eg,
    - solubility
    - viscosity
    - charge
    - conformation
    - denaturation
    - binding sites for various bacteria, viruses and parasites.

2. It protects the glycoproteins from proteolysis.

3. It regulates the proteolytic processing of precursor proteins into to smaller residues

4. It regulates the insertion of the glycoprotein into membranes,

5. It affects the intracellular migration, sorting and secretion of glycoproteins

6. It regulates embryonic development and differentiation

7. It is involved in selecting the site of metastasis for the spread of cancer cells.

Functions served by glycoproteins include:

1. Specific glycosylation pattern essential for the migration of immune cells:

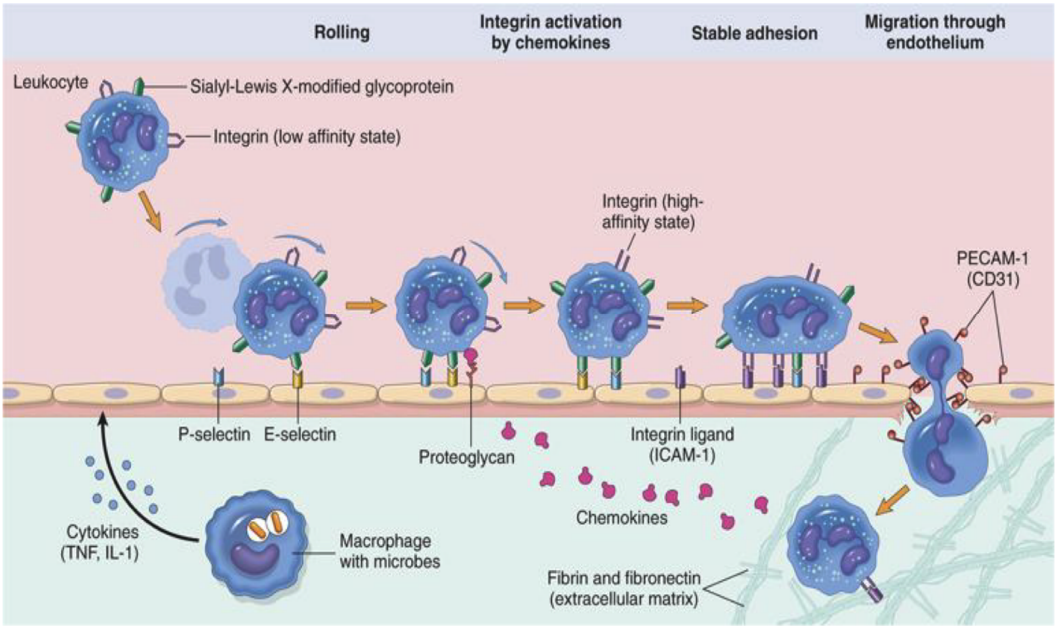
   a) N-glycans on the immune cell directs them to migrate to the appropriate site.Intercellular adhesion of specific cells such as leukocytes and endothelial cells is due to the presence of specific glycoproteins on their surfaces called selectins. Selectins are calcium binding transmembrane proteins with a number of domains. They are single chain molecules whose aminoterminal ends comprises the lectin domain.The lectin domain has its specific carbohydrate residues.

The intercellular adhesion of immune cells such as neutrophils to the endothelial cells of venules occurs in 3 stages:

1. When the endothelium is injured or inflammation occurs at the site, the neutrophils will slowly roll within the vessel wall .This event is mediated by the selectins.
2. Interaction between the activated CD18 integrin present on the neutrophils with the ICAM1 present on the endothelial cells leads to the adhesion of the activated neutrophils to the endothelium..
3. Later with the help of PECAM1 the neutrophils will migrate through the junction of the endothelial cells into the interstitium.

   b) The glycosylation pattern on the immunoglobulins alter their affinities to immune receptors.

Figure 8: Intercellular Adhesion Of Immune Cells



(Courtesy: untitled illustration of immune cells migration, Retrieved on September 5,2016. From https://www.studyblue.com/notes/note/n/case-2/deck/2532289)

c) The major histocompatibility complex (MHC) are glycoproteins present on the surface of cells and interact with T cells producing an adaptive immune response.

d) Leukocytes contains sialyl Lewis X antigen (glycoproteins) which plays an important role in cell-cell recognition on their surface.

e) Blood group antigen such as H antigen is a glycoprotein.

f) Factors IIb/IIIa responsible for platelet aggregation and adherence to the surface of endothelial cells is the glycoprotein.

2. Affects folding of certain proteins through calnexin/ calreticulin cycle.

3. Involved in the fertilization of oocyte:

- Zona Pellucida (ZP) is a thick, noncellular, transparent layer that surrounds the oocyte.
- To enter the oocyte, the sperm has to traverse this layer.
- The zona pellucida cosists of three glycoproteins namely, ZP-1, ZP-2, ZP-3, of which ZP3, which is an O linked glycoprotein is responsible for the attachment of sperms.
- Galactosyltransferase is a protein present on the surface of the sperm, which interacts with the carbohydrate chains of ZP3 inducing an acrosomal reaction. The acrosome of the sperm contains enzymes such as proteases and hyaluronidases which gets released. Release of these enzymes will help the sperm to traverse the zona pellucida and enter the plasma membrane of the oocyte.

- Fertilization can be inhibited by development of drugs and antibodies that hinder the functions of ZP3.This would thus act as contraceptive agent.

4. Role of glycoprotein in polyspermy block: The GPI protein IZUMO1R/JUNO (named after the Roman goddess of fertility) released from the plasma membrane of egg does not allow the sperm to fuse with the egg. This mechanism contributes to the polyspermy block at the plasma membrane in eggs.
5. Both *N*-linked and *O*-linked glycoproteins are present as structural components in the cell wall and extracellular matrix and help to connect together the cells and ground substance of the connective tissue. Mediates Cell to Cell and Cell Matrix interactions.
6. *O* linked glycoproteins form component of mucosal secretions which gives the mucus its slimy feel.
7. Mannose-6-phosphate is a chemical marker which targets the lysosomal enzymes to the organelle.
8. Glycoproteins play a significant roles in the human physiology as
    - Hormones- FSH, LH, TSH, HCG, Erythropoietin (EPO).
    - Alpha-fetoprotein
    - Transport molecules- Transferrin ,Ceruloplasmin
    - Enzymes- Alkaline phosphatase
    - Receptors- various proteins involved in hormone and drug action.

Factors that regulate the glycosylation of glycoprotein:

Glycosylation is a complex process which involve large number of enzymes. Glycosylation involves approximately 1 % of the human genome .It involves about ten distinct GlcNAc transferases and multiple species of other glycosyl transferases.

Factors that affect oligosaccharide assembly and transfer are:

1) Availability of appropriate acceptors on the proteins.
2) Presence of tissue Dolichol phosphate.
3) Oligosaccharide: protein transferase activity.
4) Appropriate conditions for enzyme activity which includes :
    a) The type of the cell and the processing enzymes they contain.
    b) The type of species - since same cells obtained from different species show different types of processing enzymes.
    c) Intracellular location of the enzymes.
    d) Conformation of the protein.

Methods used to analyze glycoproteins:

Various methods available to analyze glycoproteins are: Isoelectric focusing, Capillary zone electrophoresis, High performance liquid chromatography, Treatment with suitable endo/exoglycosidase or phospholipases, Agarose-lectin column chromatography[14-20], Mass spectrometry[21], NMR spectroscopy[22], Multi-angle light scattering, Dual Polarisation Interferometry, Methylation (linkage) analysis, Periodic acid-Schiff stain, Amino acid or cDNA sequencing.

CONGENITAL DISORDERS OF GLYCOSYLATION

"Congenital disorders of glycosylation (also known as carbohydrate-deficient glycoprotein syndrome) is an inborn error of metabolism in which glycosylation of a variety of tissue proteins and/or lipids are deficient or defective"[23].

In other words "Congenital disorders of glycosylation (CDG) is an umbrella term for a rapidly expanding group of rare genetic, metabolic disorders due to defects in complex chemical process known as glycosylation"[23] and is also known as CDG syndrome.

CDG is an autosomal recessive condition[24] that presents with a broad clinical presentation affecting multiple organ system. The range of severity is wide. It can be a mild disorder with asymtomatic patients or it can be even fatal leading to death.

Moderate and severe CDGs usually present at infancy itself.

Even though it is a multisystem disorder, in most of the CDGs neurological symptoms predominate[25].

Till now approximately about 100 different types of CDG have been identified.

Classification of CDG[23,26]:

(CDG) are classified into three groups:

Group I: defect in the formation of lipid linked oligosaccharide and its transfer to a nascent glycoprotein.

Group II: defect in the processing of the nascent glycoprotein.

Group X: defects not yet completely characterized

Congenital disorder of N-linked glycosylation classification (as of June 2001):

Group	Defect and localization(cytoplasm,endoplasmic reticulum,golgi apparatus)	Defective gene	Synonym
I	Defect in the assembly of dolicholpyrophosphate linked oligosaccharide chain		CDG-I
	PMM(C)	PM2	CDG-Ia
	MPI(C)	MPI	CDG-Ib
	ALG6(ER)	ALG6	CDG-Ic
	ALG3(ER)	DPM1	CDG-Id
	Dolichol-P-mannose synthase I(ER)	DPM1	CDG-Ie
II	Defect in the processing of protein bound oligosaccharide chain		CDG-II
	N-acetylglucosaminyltransferase II(GA)	MGAT2	CDG-IIa
	Glucosidase I(ER)	GLS1	CDG-IIb
	GDP-fucose transporter(GA)	FUCT1	CDG-IIc
X	Defects not yet completely characterised		

The drawbacks of older classification is that

- It is based on the order of discovery of the types of CDG disorder,
- It is a very complex classification which adds no clinical value.

Therefore this has led to the proposal of new classification by J. Jaeken in August 2008.

New nomenclature for CDG:[27]

1) Defects in protein *N*-glycosylation
2) Defects in protein *O*-glycosylation
3) Defects in glycosphingolipid and glycosylphosphatidylinositol anchor glycosylation
4) Defects in multiple glycosylation and other pathways

1. Defects in protein N-glycosylation:

CDG subtype	Gene mutation	Enzyme defect
CDG-Ia	PMM2	Phosphomannomutase 2
CDG-Ib	MPI	Phosphomannose isomerase
CDG-Ic	ALG6	glucosyltransferase 1
CDG-Id	NOT56L	mannosyltransferase 6
CDG-Ig	ALG 12	mannosyltransferase 8
CDG-Ih	ALG 8	glucosyltransferase 2
CDG-Ii	ALG2	mannosyltransferase 2
CDG-Ij	DPAGT1	UDP-GlcNAc:Dol-P-GlcNAc-P transferase
CDG-Ik	HMT1	mannosyltransferase 1
CDG-Il	DIBD1	mannosyltransferase 7-9
CDG-In	RFT1	Flippase of Man5GlcNAc2-PP-Dol
CDG-Iia	MGAT2	N-acetylglucosaminyltransferase 2
CDG-Iib	GLS1	Glucosidase 1

2. Defects in protein O-glycosylation:

O-xylosylglycan synthesis (multiple cartilaginous exotoses)	EXT1/EXT2 B4GALT7	Glucuronyltransferase/N-acetylglucosaminyltransferase b-1,4-galactosyltransferase 7
O-N-acetylgalactosaminylglycan synthesis (familial tumoral calcinosis)	GALNT3	Polypeptide N-acetylgalactosaminyltransferase 3
O-xylosyl/N-acetylgalactosaminylglycan synthesis (Schneckenbecken dysplasia)	SLC35D1	Solute carrier family 35 member D1
O-mannosylglycan synthesis cong. muscular dystrophy	POMT1/POMT2 POMGNT1 FKTN FKRP LARGE	Protein-O-mannosyltransferase 1/proteinO-mannosyltransferase 2 Protein-O-mannose b-1,2-N-acetylglucosaminyltransferase Fukutin Fukutin-related protein N-Acetylglucosaminyltransferase-like protein
O-fucosylglycan synthesis (spondylocostal dysostosis type 3) (Peters plus syndrome)	SCDO3 B3GALTL	O-Fucose-specific b-1,3-N-acetylglucosaminyltransferase O-Fucose-specific b-1,3-glucosyltransferase

3. Defects in glycosphingolipid and glycosylphosphatidylinositol anchor glycosylation:

Amish infantile epilepsy	SIAT9	GM3 synthase
glycosylphosphatidylinositol deficiency	PIGM	Phosphatidylinositolglycan, class M

4. Defects in multiple glycosylation and other pathways:

CDG-Ie	DPM1	Dol-P-Man synthase 1
CDG-If	MPDU1	Man-P-Dol utilization 1
CDG-Iid	B4GALT1	b-1,4-galactosyltransferase 1
hereditary inclusion body myopathy	GNE	UDP-GlcNAc epimerase/kinase
CDG-Iif	SLC35A1	CMP-sialic acid transporter
CDG-Iic	SLC35C1	GDP-fucose transporter
CDG-Im	DK1	Dolichol kinase
CDG-Iie	COG7	Golgi complex 7
CDG-Iig	COG1 Golgi complex 1	COG8 Golgi complex 8
cutis laxa type II	V-ATPase ATP6VOA2	V0 subunit A2 of vesicular H(+)-ATPase

Pathophysiology of CDG (*N*-glycosylation pathway)

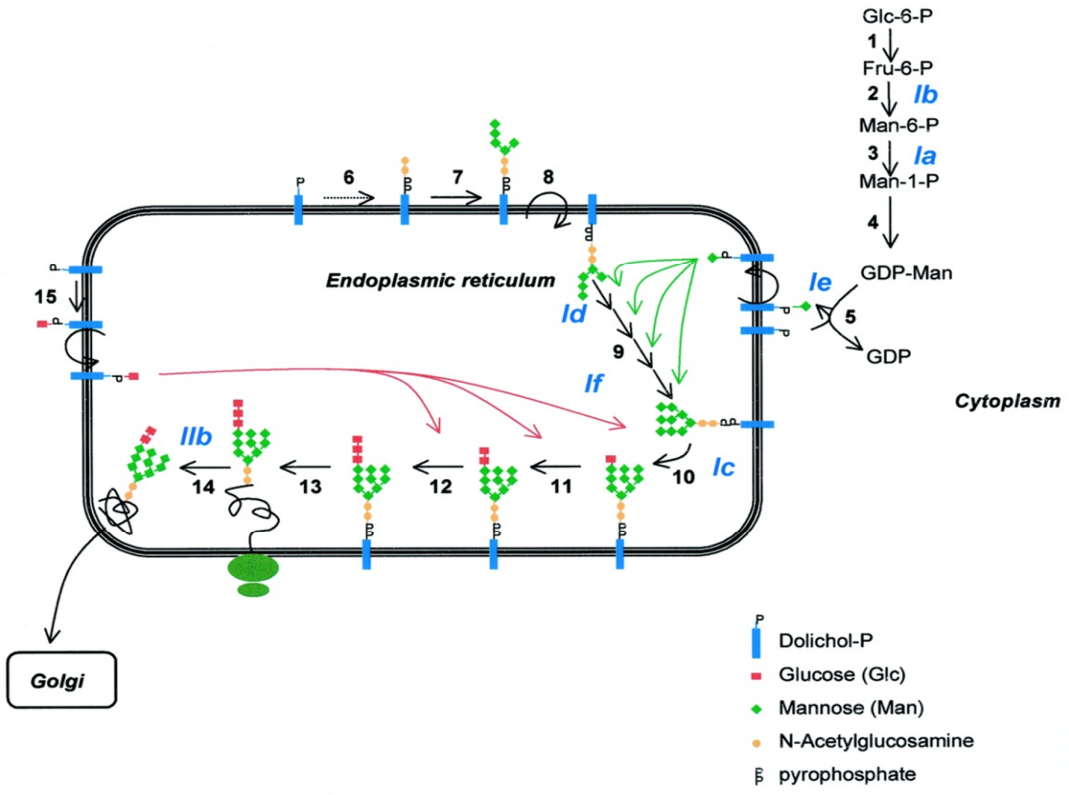
In congenital disorder of glycosylation, defect in glycosylation is due to mutatant gene which encodes for enzymes involved in glysosylation. Deficient or defective glycosylating enzymes leads to the production of defective glycoproteins which causes wide range of symptoms involving multiple organ systems.

The figure illustrates the *N*-Linked glycosylation pathway defects in CDG.

- *N*-glycosylation pathway take place in the cytoplasm and endoplasmic reticulum.
- In the cytoplasm glucose is converted into mannose which is then subsequently converted to GDP mannose.
- GDP mannose is converted into dolichol phosphate mannose (reaction 5) and UDP glucose is converted into dolichol phosphate glucose(reaction 15).
- Both the dolichol phosphate sugars are then flipped from cytoplasm to endoplasmic reticulum by the enzyme flippase.
- Key enzymes in this pathway are: (1) phosphoglucose isomerase; (2) MPI; (3) PMM; and (4) GDP-Man synthase [28].
- one molecule of GlcNAc-1-phosphate is transferred from UDP-GlcNAc to dolichol phosphate to form dolichol pyrophosphate GlcNAc,
- To dolichol pyrophosphate GlcNAc, one molecule of GlcNAc is added to form dolichol pyrophosphate $(GlcNAc)_2$ (reaction 6).
- To dolichol pyrophosphate $(GlcNAc)_2$ Five Man residues are added to form dolicholpyrophosphate$(GlcNAc)_2(Man)_5$ (reaction 7),

- To dolicholpyrophosphate $(GlcNAc)_2$ $(Man)_5$ four mannose residues and three glucose residues(reactions 10, 11, and 12) are added to form dolicholpyrophosphate Glc3Man9GlcNAc2 . These mannose residues and glucose residues are obtained from dolichol phosphate mannose (reaction 9) and dolichol phosphate glucose.
- From dolicholpyrophosphate Glc3Man9GlcNAc2, The Glc3Man9GlcNAc2 oligosaccharide is transferred to the asparagine residue of the nascent glycoprotein by the enzyme oligosaccharyltransferase (reaction 13)
- The three Glc residues are then removed from the nascent glycoprotein by glycosidases.
- Following which protein proceeds to the Golgi apparatus for further processing.
- Defective enzymes at steps 3, 2, 10, 9, and 5 causes CDG types Ia, Ib, Ic, Id, and Ie respectively.

Figure 9: Outline of the *N*-glycosylation pathway and the enzyme defects in CDG subtypes:



(Courtesy: Untitled illustration of *N*-linked glycosylation pathway defects,Retrieved on september 6, from http://www.nature.com/pr/journal/v52/n5/fig_tab/pr2002233f1.html)

Clinical features of CDG [28]

CDG-Ia (PMM deficiency):

- CDG-Ia is the most common type of CDG with approximately about 300 patients all over the world. It mostly presents during the neonatal /childhood period.
- The typical clinical features of CDG include inverted nipples, fat pads, muscular hypotonia, strabismus, failure to thrive and elevated transaminases.
- The most common clinical feature is cerebellar hypoplasia
- "As a rule there is osteopenia" [29,30]
- It is due to mutation of PMM2 located on chromosome 16p13 [31,32] .
- Mortality of this subtype is about 25%, which occurs mostly during the early childhood due to infections or organ failures [33,34].
- As the child grows, neurlogical symtoms such as mental retardation, retinitis pigmentosa ,seizures, strokelike episodes & cerebellar dysfunction become more obvious
- In adults, nonprogressive ataxia, mental retardation, and peripheral neuropathy are more common**.** Therefore most of them are wheelchair bound.
- Hypergonadotropic hypogonadism is the frequent symptom in adult female pateints.[35,36]**.**

Clinical stages of CDG-Ia[37]:

Stage1-Infantile alarming multisystem involvement:

Failure to thrive

Floppiness

Psychomotor retardation

Abnormal subcutaneous fat pads

Inverted nipples

Hepatomegaly & liver dysfunction

Cerebellar atrophy

Esotropia

Pericardial effusion

Multisystem failure

Stage 2-Childhood ataxia mental retardation:

Mental retardation IQ 40-60

Motor disability

Peripheral neuropathy

Cerebellar ataxia

Retinal degeneration

Stroke like episodes

Seizures

Stage 3-Teenage leg atrophy:

Atrophy of lower limbs

Stable crebellar ataxia

Deformity of thorax and spine

Osteopenia

Absence of puberty in females (hypergonadotrophic hypogonadism)

Stage 4-Adult stable disability:

Stable neurological impairment

Short stature

Kyphoscoliosis and long thin extremities

Premature aging

Retinitis pigmentosa

CDG-Ib (MPI deficiency):

- This disorder is due to MPI deficiency.
- Approximately about 20 CDG-Ib patients are present worldwide.
- The most comon clinical presentation include Proteinlosing enteropathy, congenital hepatic fibrosis, coagulopathy, vomiting and hypoglycemia.
- This type of CDG present without overt neurologic manifestations [38-42].
- It is one of the treatable CDG subtype with known therapy i.e. oral mannose therapy.
- Early diagnosis is indispensable.

CDG-Ic (ALG 6 deficiency):

- This disorder is due to ALG 6 deficiency, this enzyme catalyzes the attachment of the first glucose to Man 9GlcNAc2PPdolichol in the ER.

- CDGIc causes mainly a neurologic disorder similar toCDG-Ia but features such as cerebellar hypoplasia, fat pads, polyneuropathy, and inverted nipples are missing [43,44].
- The symptoms of CDGIc are general milder than CDG-Ia [45,46].
- Since the typical morphological features and radiological findings such as cerebellar hypoplasia are absent, this type is mostly underdiagnosed.
- In a very few patients the disease lead to a fatal outcome.

CDG-Id (ALG 3 deficiency):

- The defect is due to ALG 3 which transfers mannose from dolichol - phosphate mannose to the lipid linked oligosaccharide intermediate Man5GlcNAc2PPdolichol.
- In 1995, a child presented with microcephaly, severe epilepsy, global developmental delay and psychomotor retardation. It was first reported as CDG "type IV" [47].
- The isoelectric focusing of serum transferrin showed no increase of asialotransferrin.
- Till now only one patient is identified as ALG3 deficiency [48].

CDG-Ie (dolichol-P-mannose synthase 1 deficiency):

- CDG-Ie is caused by DPM1 gene mutation which leads to dolichol-P--mannose synthase deficiency (EC 2.4.1.83) [49,50]

- Four patients are reported with clinical features such as microcephaly, minimal psychomotor development, absent visual contact and severe epilepsy.
- In two of the above said 4 pateints MRI brain revealed delayed myelination
- Serum transferrin electrophoresis by isoelectric focusing showed only little or no increase of asialotransferrin.

CDG -If:

It is due to mutations involving Lec35/MPDU1 gene leading to deficiency or defective dolichylphosphomannose and dolichyl phosphoglucose. Clinical features of this disorder include severe encephalopathy, dry scaling skin with erythroderma.

CDG-IIa (*N*acetylglucosaminyltransferase II deficiency):

- CDG-IIa is due to mutations in MGAT2 gene which codes for the enzyme N-acetylglucosaminyltransferase II [51].
- In the early 1990s two children were reported with cerebellar hypoplasia and severe psychomotor retardation.
- But these patients did not have peripheral neuropathy.
- Till now, four children have been identified as CDG-IIa [52,53].
- Clinical features of this type include psychomotor retardation, craniofacial dysmorphy and stereotypic hands movement.
- Glycan structural studies revealed a glycan pattern similar to congenital dyserythropoietic anemia type II [54,55].
- *N*-acetylglucosaminyltransferase II deficiency was demonstrated in monocytes and fibroblasts [56,57].

CDG-IIb (glucosidase I deficiency):

- This type of CDG is due to glucosidase enzyme deficiency.
- Signs and symptoms of CDG-IIb include typical dysmorphic features such as retrognathia, high arched palate, and overlapping of fingers.
- Other clinical features of this subtype include severe developmental delay, seizures, muscular hypotonia and recurrent edema.
- Though serum transferrin electrophoresis by isoelectric focusing was normal, Isoelectric focusing of serum hexosaminidase revealed the abnormality.
- The diagnosis of this disorder was by oligosaccharide analysis of urine which showed the presence of a Glc (α1–2) Glc (α1– 3) Glc (α1–3) Man [58].

CDG-IIc (GDP-fucose transporter deficiency):

- This disorder is due to GDP-fucose transporter gene mutation
- This disorder is also called as leukocyte adhesion deficiency type II [59,60]
- Till now three patients have been reported [61,62]
- Clinical features include, severe psychomotor retardation, growth retardation, hypotonia, and craniofacial dysmorphism.
- Neutrophils of these patients do not have Sialyl lewis x which is a carbohydrate containing molecule and responsible for cell adhesion. Sialyl Lewis x is required for the recruitment of neutrophils to the site of infection. Therefore the patients present with recurrent infections and marked leukocytosis.
- Other fucose containing carbohydrate structures, such as A, B, O, and Lewis A blood groups, are also absent.

CDG-IId:

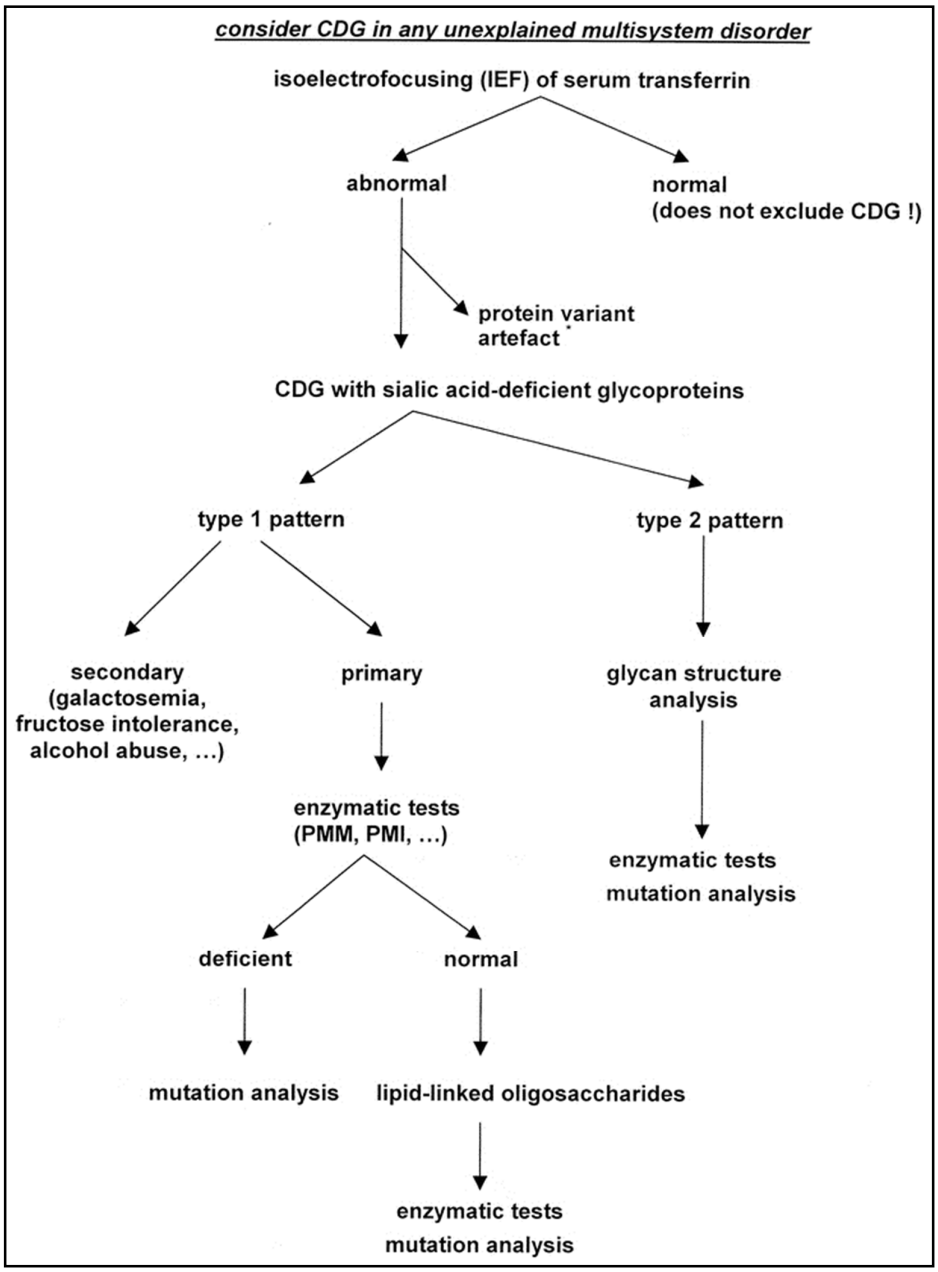
- Due to deficient UDP-Gal: N-acetyl-glucosamine -1,4-galactosyltransferase I enzyme
- Clinical features include psychomotor retardation, macrocephaly and myopathy.

CDG-x:

- The number of CDG patients that cannot be included in the above mentioned subtypes is progressively increasing.
- They may be due to different defects in the various other steps of the *N* glycosylation and *O* glycosylation pathways.
- They present with wide range of clinical presentation such as : absent psychomotor development, progressive microcephaly, oligohydramnion, hypotonia,failure to thrive, hydrops fetalis, dysmorphy, early death after intractable seizures, [63]

  Severe hypotonia, cataracts, death in status epilepticus [64], oligohydramnios, dysmorphy, hypotonia, diarrhea, vomiting, ascites, seizures, cerebellar hypoplasia, severe thrombocytopenia [64], muscular hypotonia, infantile spasms [65] .demineralization of distal bones, tubulopathia [66], dysmorphy,

Figure 10: Algorithm for the diagnosis of CDG[30]



(Courtesy**:** Congenital Disorders of Glycosylation: A Review Stephanie Grünewald, Gert Matthijs and Jaak Jaeken)

DIAGNOSIS OF CDG:

Serum transferrin electrophoresis using isoelectric focusing is the gold standard technique in the diagnosis of congenital disorder of glycosylation.

Human Serum Transferrin

- Transferrin belongs to the class of single chain iron binding glycoprotein. 6 % of the glycoprotein contains carbohydrate. It is a β 1 globulin whose molecular mass is about 80 KDA with 678 amino acids [67]. Interestingly, the carbohydrate moiety of transferrin is not essential for its optimum function [68].
- Apotransferrin gene is found at q21 on chromosome 3 near the gene for Transferrin receptor .Apotransferrin is produced in the liver.It has a half life of 8 days [67]. It contains 2 homologous domains, one in both N and C terminal. Each of those domain contains one high affinity fe $^{3+}$ binding site[67].
- Iron binds apotransferrin through two tyrosine molecules, an aspartic acid residue and a histidine residue. An anion (bicarbonate or carbonate) is bound with each ferrin ion as a bridging ligand between protein and the iron [67]. Affinity of apotransferrin to iron is a pH dependent process. In plasma with pH 7.4 apotransferrin very strongly binds the iron. But at pH less than 4.5 no binding occurs. This pH dependent affinity of transferrin play a significant role in the mechanism of iron release from transferrin under physiological conditions.

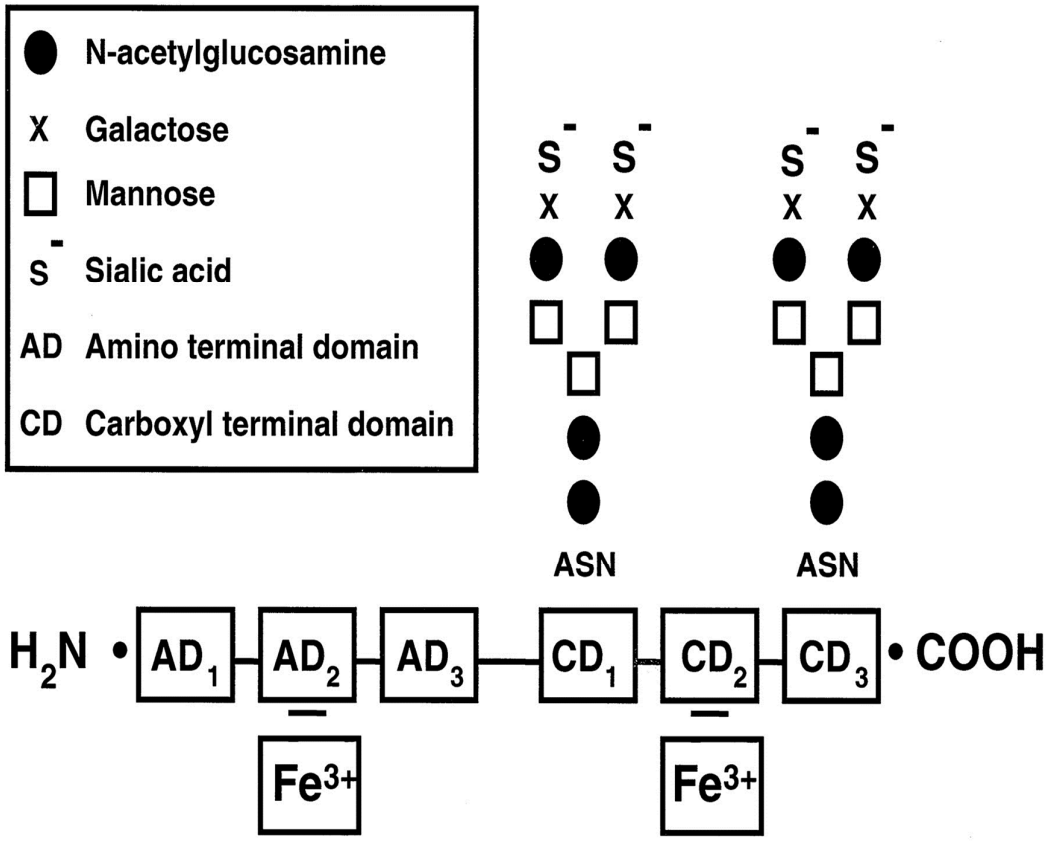
- Conformational changes in the protein leads to release & binding of iron by transferrin. In case of absence of iron, the two domains of transferrin are widely separated and it assumes an open configuration. However when iron binds to the transferrin the domains come close to the metal to assume a closed configuration state.

The reference range of serum transferrin: 200 to 400 mg/dl

Conditions causing increased transferrin levels:Iron depletion, Pregnancy, Oral contraceptives

Conditions causing decreased transferrin levels: Ironoverload, Inflammation, Infection, Malignancy, Liverdisease, Nephritic syndrome and Malnutrition.

Figure 11 : Structure of transferrin



(Courtesy: untitled illustration of transferrin structure, Retrieved on August 10,2016 from http://www.medicine.org.hk/hkscc/communication/v5n1/p23-26f.htm)

Microheterogeneity of human serum transferrin:

Transferrin is synthesized by the hepatocytes and is made up of three domains

i. A single polypeptide chain

ii. Two metal ion binding sites

iii. Two N linked complex glycan chains.

Transferrin shows distinct microheterogeneity. Even under nonpathological condition the transferrin substructures show distinct variability.

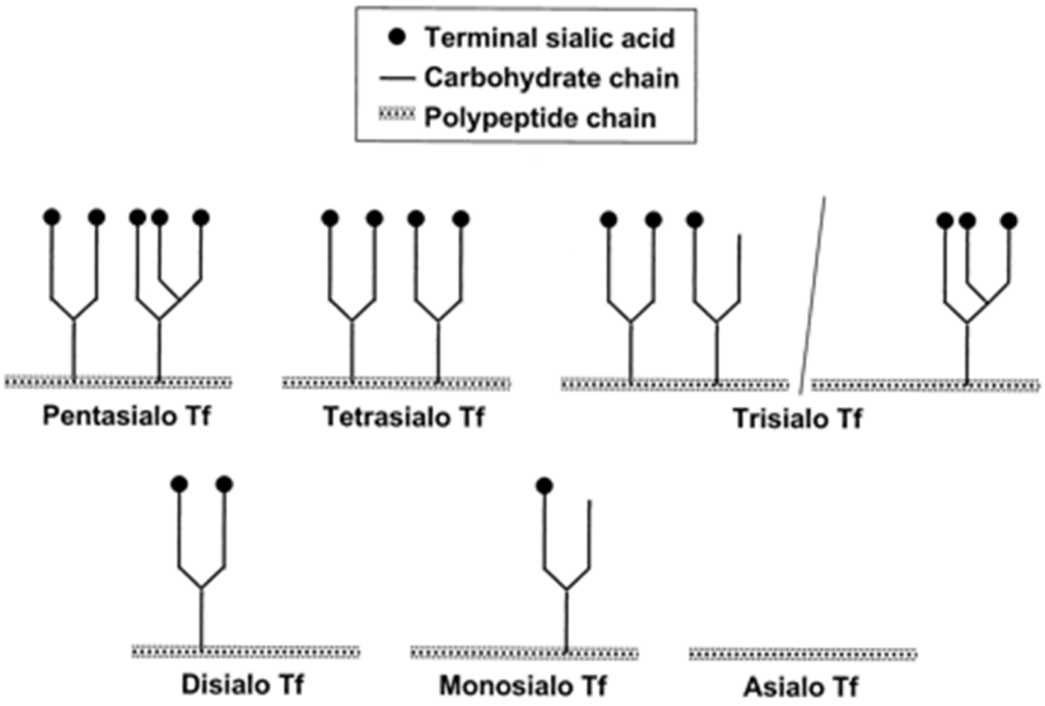
Human serum transferrin appears in the β region of serum protein electrophoresis on agarose gel. A multitude of transferrin bands appears in isoelectric focusing with immunoblot and staining.

Effect of varying iron load:

Each Transferrin molecule can bind with two metal iron, preferably Fe^{3+}. Depending upon the availability of Fe^{3+}, Transferrin molecules can be Iron free, Loaded with one Fe^{3+} ion (Fe_1-Transferrin; Fe_1N or Fe_1C transferrin), Loaded with two Fe^{3+} ions(Fe_2-transferrin). In healthy individuals, transferrin iron saturation is 30%. In Fe^{3+} deficient state, iron saturation of transferrin decreases and increased levels of Fe_0 and Fe_1-transferrin is found in the serum. In Fe^{3+} excess state (hemochromatosis), iron saturation of transferrin increases and only Fe^{3+} isoform is found in the serum.

The isoelectric point of the transferrin molecule decreases by 0.2 pH units for every Fe^{3+} ion binding.

Figure 12: Transferrin isoforms



(Courtesy: untitled illustration of transferrin isoforms ,retrieved on september 9,2016 from http://www.ufrgs.br/imunovet/molecular_immunology/chemicalcauses_ethanol.html)

Differing *N*-glycan chains

The two *N*-glycan chains in the transferrin molecule differs in their degree of branching with bi, tri and tetra antennary structures. Each antenna of the transferrin *N*-glycan chain terminates with a negatively charged sialic acid residue.Because of this, sialoisoforms of transferrin (i.e.asialotransferrin, monosialo,disialo,trisialo,tetrasialo,pentasialo,hexasialo, heptasialo,octasialo transferrin) occurs in the serum. The relative amount of this transferrin isoforms as a percentage of total serum transferrin is :

Transferrin sialo isoform	Relative percentage
Heptasialotransferrin	<1.5%
Hexasialotransferrin	1-3 %
Pentasialotransferrin	12-18%
Tetrasialotransferrin	64-80%
Trisialotransferrin	4.5-9%
Disialotransferrin	<2.5%
Monosialotransferrin	<0.9%
Asialotransferrin	<0.5%

The octasialotransferrin is not in detectable amount in the serum under physiological condition. The isoelectric point of transferrin decreases by 0.1 pH unit for each sialic acid residue bound to the *N*-glycan chain. Genetic variants of transferrin is possible due to substitutions of aminoacid in the polypeptide chain Till now about 38 transferrin variants are known.

The factors affecting isoelectric point of the transferrin molecule are:

1. Ironload
2. Amount of sialic acid
3. Polypeptide chain polymorphisms

The alterations in isoelectric point is possible. When one or two Fe^{3+} ions are bound or lost, it can be compensated either by the presence or absence of sialic acid residues or by genetic variants of transferrin. Thus, transferrin molecules with differing iron content and sialic acid residues can have the same isoelectric point.for eg, disialo Fe_2-Transferrin which is the main CDT isoform and terasialo Fe_1-Transferrin which is the main non CDT isoform can have the same isoelectric points.

Isoelectric focusing (IEF):

IEF separates amphoteric compounds such as proteins with increased resolution in a medium possessing a stable pH gradient. The protein becomes focused at appoint on the gel as it migrates to a zone where pH of the gel matches the protein's isoelectricpoint. At this point, the charge of the protein becomes zero and its migration ceases. The protein zones are very sharp, because the region associated with a given pH is very narrow. Proteins that differ in their pI values by only 0.02 pH units have been separated by isoelectric focusing.

In Isoelectric focussing mixtures of 50 to 100 different ampholytes with molecular weights of 300 to 1000 and different pKa values are added to the medium to create a natural pH gradient through which the proteins migrate and stop at their individual isoelectric points.

PAGE-IEF: It is widely used as it is free of electroendosmosis. The pore size of the polyacrylamide gel must be large enough so that protein migration is not impeded by molecular sieving effects. In actual practice, impeded migration of some proteins, such as IgM, cannot be prevented. With the availability of endosmosis free material, IEF methods have been adopted for agarose, cellulose acetate, and sephadex electrophoresis.

Isoelectric focusing of serum Transferrin:

Transferrin, a serum glycoprotein has two *N*-glycosylation sites.Tetrasialotransferrin being the common sialoisoform in the serum .It consists of two biantennary chains with sialic acid residues in their terminal end. When synthesis of *N*-glycan is deficient, the incorporation of sialic acid residue which is a negatively charged residue becomes deficient.This leads to the acquisition of a more positive charge in the molecule. This leads to a cathodal shift in the isoelectric focusing of transferrin.Two types of abnormal patterns are commonly found in isoelectric focusing of serum transferrin. In type 1 pattern/pattern A (the most frequent pattern), there is a decrease in tetrasialotransferrin and increase in di and asialotransferrin. In type 2 pattern/ pattern B there is a combination of type 1 pattern along with increase in trisialo/monosialotransferrin.It is ideal to exclude that the abnormal pattern is not due to transferrin protein variant by performing Mass spectrometry of the patient's serum transferrin or atleast by preincubating the sample with neuraminidase.IEF of other glycoproteins such as hexosaminidase, Thyroxine binding globulin and α-1 antitripsin, apo C-III helps to document the presence of generalized glycosylation defect in the pateint. The disadvantage of IEF is that defects in fucose cannot be diagnosed using IEF since isoelectric focusing is based upon sialic acid deficiency. This could be overcome by identifying the presence of Bombay blood group in the patient who is suspected to have a fucose defect. IEF was normal in one patient with CDGIIb [54].IEF of serum transferrin can also be normal in pateints with CDGIa [69].

Capillary zone electrophoresis of serum Transferrin an alternative to isoelectric focussing[70]:

Capillary zone electrophoresis

In Capillary zone electrophoresis (CZE), the classic techniques of zone electrophoresis, isotachophoresis, isoelectric focusing and gel electrophoresis can be carried out in a small bore fused silica capillary tube of 10-100 μm internal diameter and 20 to200 cm in length.

Two distinct advantages of capillary format are Higher voltage can be applied thereby migration of proteins can be made fast and Possibility of automation

CZE can be used to separate low molecular weight ions and uncharged molecules using the micellar electrokinetic chromatography (MEKC) mode.CZE can also be used for separation of inorganic ions, amino acids,organic acids, drugs, vitamins, porphyrins, carbohydrates, oligonucleotides, proteins and DNA fragments.

In an electrophoretic apparatus, the capillary tube is connected to buffer reservoirs to which a high voltage power is supplied. This helps to obtain enhanced resolution and decreases the separation time. The capillary tube is connected to a detector at its end.The technique requires only few microlitres of sample. This minimizes distortions caused by the presence of excess of samples in the applied field. In contrast to the conventional electrophoresis, CZE is well

suited to automation. Samples are easily applied to the capillary, a variety of detector types can be used.

The capillary tubes:

The capillary tubes used in CZE are made from fused silica covered with a thin polyimide coating which provides the tube strength and flexibility.. At the terminal end of the tube a small portion of the polyimide coating is removed, creating a window for online optical detection. The length of the capillary tubing is from 20 cm upto several metres with the outer diameter being 180 to 375 μm and inner diameter being 20 to 180μm. Rectanglar capillaries are more suitable for optical detection than their cylindrical counterparts as they provide a flat surface. The main advantage of performing electrophoresis in a narrow bore capillary is efficient heat dissipation. The large surface to volume ratio inherent in capillaries, however allows for more effective heat dissipation and much higher voltages can be applied, often upto 30 kV.An additional advantage gained from using a narrow bore capillary chamber is that it improves resolution by minimizing band broadening.

Sample injection:

In CZE, sample volumes of less than few microlitres (1 to 50 nL) are loaded into the capillary chamber by one of two primary methods. Which are Hydrodynamic injection and Electrokinetic injection.

With hydrodynamic injection, the sample is applied by a positive pressure at the inlet or negative pressure at the outlet..

In electrokinetic injection, the sample is applied by a voltage for a timed interval. The magnitude of voltage used for sample application is usually 3 to 5 times lower than the voltage used for separation.

Direct detection:

With CZE, the analytes are separated fast. Detection is by an optical detector without prior staining. Optical detection is based on classical methods, such as photometric absorbance, refractive index, and fluorescence.

Optical detection:

As with HPLC, ultraviolet visible photometers are widely used as detectors, the inner diameter of the capillary tube defines the optical light path (OLP) length of the tube.

This Capillary zone electrophoresis system can be used to screen patients suspected with congenital disorder of glycosylation by estimating and interpreting the carbohydrate deficient transferrin pattern.

Carbohydrate Deficient Transferrin

Transferrin isoforms, such as asialo Fe_2-Transferrin, monosialo Fe_2-Transferrin, disialo Fe_2-Transferrin were collectively known as carbohydrate deficient transferrin. The normal range for carbohydrate deficient transferrin is <1.3 %

Trisialo Fe_2-Transferrin and CDT:

Trisialo Fe_2-Transferrin has no obvious diagnostic value and it should not be considered in CDT. This is because the concentration of Trisialo transferrin was exactly same in a pathological sample when compared to a non pathological serum samples having normal CDT concentration [71].

Structure of human serum CDT:

The main CDT isoforms are disialo Fe_2-transferrin and asialo Fe_2-Transferrin. They lack one or two complete glycan chains. The disialo Fe_2-transferrin has a single biantennary Nglycan chain with two sialic acid residues, where as asialo Fe_2-transferrin consists of no carbohydrate structure therefore no sialic acid residues.

Trisialo Fe_2.transferrin has two biantennary N-glycans, one with two terminal sialic acid molecules, the other with one terminal sialic acid and one terminal galactose.

Pentasialo Fe_2-transferrin contain one biantennary, disialylated N glycan and one triantennary trisialylate N-glycan. The structure of monosialo-Fe_2-

transferrin, which is a part of CDT, remains unclear. The CDT isoforms can have either deficient or defective N glycan chains.

In contrast to other glycoproteins, lack of terminal sialic acid residues in the CDT isoforms does not affect hepatic clearance of CDT through the asialoglycoprotein receptor.

The plasma half life of CDT is 14 days whereas the plasma half life of transferrin is 7 days [72] .

CDT and alcoholism:

Decreased sialic acid content is found in serum samples of alcoholic pateints. The samples showed normal transferrin isoform patterns after the complete removal of sialic acid residues by treatment with neuraminidase. Thus serum transferrin from alcoholics showed sialic acid deficient transferrin carbohydrate structure.

Further study revealed lack of links in N glycan chains in addition to absent sialic acid residues.

Other causes for increased CDT include fructosemia, galactosemia and in chronic alcoholism[73-77]

OTHER TECHNIQUES USED FOR THE DIAGNOSIS OF CONGENITAL DISORDER OF GLYCOSYLATION:

a. Enzymatic studies:

Since the principal defect of CDG lies in the deficient or defective enzymes that are required for glycosylation, enzymatic studies confirms the subtype of CDG. Phosphomannomutase, the enzyme deficiency in type CDGIa can be measured in both fibroblast and leukocytes. However enzyme activity measurement in leukocytes is found to be more reliable than measuring enzyme activity measurement in fibroblast. This is because enzyme activity in leukocytes were always in an abnormal range when compared to fibroblasts that had a high residual enzyme activity [78].

b. Mutation studies:

In 1997, the phosphomannomutase 2 gene was cloned and more than 50 different mutations [79-83] was identified of which missense mutation was the predominant one. The most common mutations are mentioned below [84-88]

Gene	Frequent mutations	Other mutations
PMM2 (CDG-Ia)	R141H,F119L,V231 M,P113L	C9Y,D65Y,V129M,F183S,D188G,T237M,T23 7R,C241S
MPI (CDG-Ib)	M51T,S102L,D131 N,M138T,R152Q, R219Q,	G250S,Y255C,1398T,R418H
ALG6 (CDG-Ic)	Dutch mutation A333V	IVS3+5G>A,S478P,F304S,Δ1299

Till date only a handful of other mutations has been identified [89-91].

c. Lipid Linked Oligosaccharide analysis (LLO):

The defects of CDGIc, Id, and Ie can be elucidated by comparing the LLO of yeast mutant strains with the Lipid Linked Oligosaccharide structures of patients. However, LLO analysis is limited to defects in the cytosol and ER.

Prenatal diagnosis of CDG:

Prenatal diagnosis for CDGIa[92] is now possible due to the availability of

a. Mapping the disease locus on chromosome 16p13

b. Consequent identification of the enzyme defect

c. Cloning of the PMM2 gene.

Prenatal diagnosis based on transferrin IEF in fetal blood is not reliable[93,94]. Therefore Enzyme activity measurement in cultured trophoblasts or amniocytes are useful but they may also be inconclusive at times [82]. Therefore, in the fetus direct mutation analysis is preferred.

Prenatal diagnosis of CDG is possible, only if it satisfies one of the two below mentioned conditions

1) The diagnosis has been confirmed in the index patient
2) The mutations have been detected in the parents.

MANAGEMENT OF CDG [95-100]:

At present only three forms of CDG have specific treatment. They are CDG-Ia, CDG-I b, CDG-IIc

CDGI-a: mannose therapy and fucose supplementation to enhance GDP-mannose pool was not much successful. It is found that glucose starvation improves N-glycosylation in fibroblasts.Based on this finding research is going regarding the influence of ketogenic diet in CDG-Ia. Acetyl salicylic acid of 0.5 mg/kg per day is given to symptomatic pateints to reduce stroke like events. Biphosphonates should be given to pateints with recurrent fractures.

CDG-Ib: Life long oral mannose supplementation at a dose of 100-150 mg/kg/day (in 4-6 doses/day) has shown significant improvement. Serum mannose is maintained above 200 M. Side effects of mannose therapy such as osmotic diarrhoea and increase in glycosylated haemoglobin should be monitored carefully.

CDG-IIc: Fucose supplementation at a dose of 25mg/kg/day in three doses improves the fucosylation of glycoproteins and controls recurrent infections.

Though other forms of CDG has only supportive treatment at present.

Research in the field of enzyme replacement therapy and gene therapy may provide specific treatment for other forms of CDG in near future.

Aim of the study

AIM AND OBJECTIVES OF THE STUDY

1) To study Transferrin electrophoresis pattern among the apparently healthy control group and clinically suspected congenital glycosylation disorders group.
2) To identify the prevalence of abnormal transferrin glycosylation in the study population

Materials & Methods

MATERIALS AND METHODS

The study protocol was approved by the Ethical committee, Madras Medical College. The copy of which is enclosed.

Study design **:** Case control study

Study period **:** January 2016 to September 2016

Subject selection:

Group 1: (Study group)

Samples were collected from 30 cases with clinical signs and symptoms strongly suspicious of congenital disorder of glycosylation.

Group 2: (Control group)

Samples were collected from 30 individuals who are apparently healthy. Individuals had no signs and symptoms of congenital disorder of glycosylation and any other chronic illness or co morbidities. Samples for the control group were obtained from both paediatric and adult population irrespective of the age or sex of the individual since glycosylation of transferrin is not affected by age or sex.

Inclusion criteria:

1. Individuals with clinical features of congenital glycosylation disorders
    a. Neurological signs-psychomotor retardation, ataxia, hypotonia, areflexia, seizures
    b. Ophthamological signs-strabismus, coloboma iris, retinopathy, optic atrophy
    c. Dysmorphic features-inverted nipples, cutis laxa
    d. Protein losing enteropathy
    e. Coagulopathy
    f. Hypogonadism
    g. Radiological findings-Cerebral/Cerebellar atrophy, Cerebellar hypoplasia

3. Unexplained neurological syndrome particularly when associated with other organ disease.
4. Unexplained syndrome even without neurological involvement

Exclusion criteria:

1. Children younger than one month due to the presence of maternal isoforms
2. H/O alcohol intake.
3. H/O liver cirrhosis

Sample collection:

Under strict aseptic precautions 2-3 ml of venous blood sample was collected in a serum separator tube.

Sample preparation:

Serum separator tube containing the venous blood sample was allowed to stand for 35 minutes in order for clotting and serum to separate. (This step is crucial since plasma fibrinogen interferes with the carbohydrate deficient transferrin assay). The sample was then centrifuged at 3000 rpm for 10 minutes. Serum was separated from the centrifuged sample and was stored in a 2 ml eppendorf tube.

Sample storage:

Serum in the 2ml eppendorf tube was stored at – 40 degree Celsius for a period of not more than one month, since the stability of the sample for the assay is up to 30 days at -40 degree Celsius

Sample analysis:

Methodology:

Serum transferrin electrophoresis using Sebia's capillary zone electrophoresis MINICAP system and carbohydrate deficient transferrin assay kit.

- This technique separates the serum transferrin isoforms into 5 fractions namely: asialotransferrin disialotransferrin, trisialotransferrin, tetrasialotransferrin and pentasialotransferrin.

- The low-sialylated isoforms- disialotransferrin, monosialotransferrin and asialotransferrin constitutes CDT (Carbohydrate Deficient Transferrin).
- The separated transferrin isoforms are detected and quantified at an absorbance of 200 nm.

Principle of the test

With this technique, charged molecules are separated by electroosmotic flow at a pH of 8.8

Electroosmotic flow (electro endosmosis): Capillaries are fixed with hydroxyl ions. Therefore the positive ions in the solution form cluster around the negatively charged hydroxyl ions. This creates a stern potential.The potential that exist between fixed and associated cloud of ions is termed as the electrokinetic potential/zeta potential.

On application of the current the fixed ions remain immobile whereas the cloud of ions move towards the oppositely charged electrode. Since the ions in solution are well hydrated, the solvent too moves along with the ions. The movement of solvent and its solutes relative to the fixed support is known as endosmosis which causes the preferential movement of water in one direction. Macromolecules in solution which move in the opposite direction will remain immobile or may even be swept backtowards if the charge on them is insufficient.As the inner surface of a glass capillary tube is coated with many charged groups, endosmosis is very strong. Endosmosis is the primary driving force for migration in the capillary electrophoresis system.

The MINICAP System has 2 capillaries functioning in parallel. Sample is diluted with the specific sample diluent and injected at the capillary's anode end. A high voltage is applied for protein separation and direct detection of the proteins is at 200 nm at the capillary's cathodic end. Following which the capillaries are washed with the wash solution.

Requirement:

1. Sebias MINICAP capillary electrophoresis system.

2. Sebias MINICAP CDT kit.

The kit contains:

a. Buffer (ready to use) 2 vials, 250 mL each

b. Sample diluent (ready to use) 1 vial, 80 mL

c. Wash solution (stock solution) 1 vial, 25 mL

d. CDT wash solution (ready to use) 1 vial, 80 mL

e. Sample treatment solution.

f. Reagent cups 1 pack of 1 25

g. Filters 3 filters

h. Bins for used cups 4 bins

Preparation of serum samples to remove interferences from immunoglobulins:

200 µL of serum is added to 50 µL of samples treatment solution (PN2054) provided in the kit. Vortexed for 5 seconds. It is then centrifuged for a minute at 3000rpm. The supernatant is collected and then analyzed.

Samples to avoid:

- Hemolyzed serum samples since Hemolysis distorts the transferrin isoforms electrophoretic pattern.
- Aged, improperly stored serum samples.
- Plasma samples as Fibrinogen migrates before the asialotransferrin isoform and causes distortion of the electrophoretic pattern.
- Samples that contain EDTA or citrate.

PROCEDURE:

1) Software for CDT analysis was installed.
2) MINICAP CDT buffer was kept in position "B2" in the instrument.
3) CDT analysis program was selected
4) Appropriate coupling cap, pipe and filter was fixed
5) New reagent cups was placed on the appropriate place in the loading system
6) New bin for used cups was placed in the intended position.
7) Reagent level was checked and in the window "Check reagent levels"was selected and updated in the software.

8) Samples was placed in the the rotating sampler which has 26 positions for sample tubes (positions No. 1 to 26)
9) Sample diluent was placed in position No. 27on the rotating sampler (Diluent Solution position).
10) Rotating sampler was slided into the MINICAP system.
11) Doors of the MINICAP system was closed which acts as a sensor for the start of the analysis.

Dilution - Migration - Description of the Automated steps

1. Samples were diluted with sample diluent and the sample probe was rinsed after each sample.
2. Capillaries were washed.
3. Diluted sample was injected into capillaries.
4. Migration was carried out under constant voltage for about 8 minutes and the temperature was controlled by Peltier effect.
5. Transferrin isoforms were detected directly by scanning at 200 nm and an electrophoretic profile appeared on the screen of the system.

Result Analysis

At the end of the analysis, system displayed electrophoretic pattern and quantified the concentration (%) of each fraction Transferrin isoforms i.e. pentasialotransferrin, tetrasialotransferrin, trisialotransferrin, disialotransferrin, asialotransferrin and CDT.

Statistical Analysis

STATISTICAL ANALYSIS

The results obtained were analysed as follows

1. Distribution of transferrin isoforms among cases and controls
2. Statistical significance in the distribution of asialo,mono,di,tri,tera,penta sialotransferrin isoforms between adult and pediatric age group in the control group was analysed
3. Statistical significane in the distribution of asialo, mono, di, tri, tera, penta sialotransferrin isoforms between males and females in the control group was analysed
4. Comparision of transferrin isoforms in various clinical presentations among the cases.
    - Consanginous vs Non consanginous
    - Similar family history vs No similar family history,
    - Dysmorphic features vs Normal appearance,
    - Ophthalmological signs vs No ophthalmological signs
    - Radiological findings vs No radiological features
5. Prevalence of CDG in the study population.

Statistical analysis was done using SPSS software version 20.0 by Independent sample T test and Chi square test. P-value <0.05 is considered as statistically significant .Confidence interval 95%.

Results

MASTER CHART

DISTRIBUTION OF TRANSFERRIN ISOFORMS AMONG THE CONTROLS:

Group	Age group	Sex	Asialo Transferrin (%)	Monosialo Transferrin (%)	Disialo Transferrin (%)	Trisialo Transferrin (%)	Tetrasialo Transferrin (%)	Pentasialo Transferrin (%)	CDT Transferrin (%)
Control	Paediatric	Male	0	0	0.7	4	84.1	11.2	0.7
Control	Adult	Male	0	0	0.9	1.2	80.5	17.4	0.9
Control	Adult	Male	0	0	0.8	1.9	82.2	12.3	0.8
Control	Adult	Female	0	0	0.7	1.5	82.6	12.3	0.7
Control	Paediatric	Female	0	0	0.7	2.2	77.8	19.3	0.7
Control	Adult	Female	0	0	0.4	3.7	80.4	16.3	0.4
Control	Adult	Female	0	0	0.9	2.2	83.2	13.7	0.9
Control	Adult	Male	0	0	0.7	2.6	81.3	15.4	0.7
Control	Paediatric	Male	0	0	0.6	0.9	85	13.5	0.6
Control	Adult	Female	0	0	0.6	1.6	83.1	12.6	0.6
Control	Paediatric	Male	0	0	0.7	3	77.8	18.5	0.7
Control	Paediatric	Female	0	0	0.8	2.3	80.7	16.2	0.8
Control	Paediatric	Male	0	0	0.7	3	82.4	13.9	0.7
Control	Paediatric	Female	0	0	0.6	4	74.7	20.7	0.6
Control	Adult	Female	0	0	1.4	3.3	81.7	13.8	1.4
Control	Adult	Male	0	0	0.5	2.6	83.4	16.5	0.5
Control	Adult	Female	0	0	0.8	3.8	81.4	14	0.8
Control	Adult	Female	0	0	0.4	1.5	83	15.1	0.4
Control	Paediatric	Female	0	0	0.8	1.6	83	14.6	0.8
Control	Adult	Male	0	0	0.6	1.7	80.3	14.7	0.6
Control	Adult	Male	0	0	0.4	1.6	82.1	14.6	0.4
Control	Adult	Male	0	0	1.1	1.7	81.3	16.6	1.1
Control	Adult	Male	0	0	1	2.6	82.8	13.6	1
Control	Adult	Male	0	0	0.4	2.7	82.8	14.1	0.4
Control	Adult	Female	0	0	0.6	1.9	80.7	13.5	0.6
Control	Paediatric	Male	0	0	0.9	2.8	80.5	15.8	0.9
Control	Adult	Male	0	0	0.8	2.7	82.8	15.7	0.8
Control	Adult	Female	0	0	0.9	2.8	81.3	15	0.9
Control	Paediatric	Female	0	0	1	2.7	80.3	16	1
Control	Paediatric	Male	0	0	1.1	2.9	80.1	15.9	1.1

DISTRIBUTION OF TRANSFERRIN ISOFORMS AMONG THE CASES:

Group	Asialo Transferrin (%)	Monosialo Transferrin (%)	Disialo Transferrin (%)	Trisialo Transferrin (%)	Tetrasialo Transferrin (%)	Pentasialo Transferrin (%)	CDT Transferrin (%)
Cases	6.6	0	0.9	1.7	77.8	13	7.5
Cases	0	0	0.7	0.7	84.9	13.7	0.7
Cases	0	0	0.7	2.7	82.4	14.2	0.7
Cases	0	0	1	3.4	82.6	13	1
Cases	0	0	0.5	3.6	84	11.9	0.5
Cases	0	0	0.7	1.4	79.9	18	0.7
Cases	0	0	0.5	1.1	83.7	14.7	0.5
Cases	0	0	0.5	1.5	81.5	16.5	0.5
Cases	13.8	0.3	52.6	2.7	24.4	6.2	66.4
Cases	0	0	0.5	2.8	82.8	13.9	0.5
Cases	0	0	0.8	1.9	82	15.3	0.8
Cases	0	0	0.3	4.1	79.2	16.4	0.3
Cases	0	0	0.8	2.7	82.7	13.8	0.8
Cases	0	0	0.9	0.2	75.7	23.2	0.9
Cases	10.2	0.2	46.2	2.4	31.9	9.1	56.4
Cases	0.4	0	0.4	1.2	81.2	16.8	0.8
Cases	0	0	0.6	3.1	86.2	10.1	0.6
Cases	0	0	0.7	3	83.6	12.7	0.7
Cases	0	0	1.1	0.8	82.2	15.9	1.1
Cases	0	0	1.1	1	79.1	18.8	1.1
Cases	0	0	0.6	1.1	81.3	17	0.6
Cases	0	0	0.6	3.9	82.9	12.6	0.6
Cases	0	0	0.7	0.8	81.3	17.2	0.7
Cases	0	0	0.5	1.7	84.1	13.7	0.5
Cases	0	0	0.3	3.2	79.2	17.3	0.3
Cases	0	0	0.9	1.8	82.9	14.4	0.9
Cases	0	0	0.7	3	82.7	13.6	0.7
Cases	0	0	0.8	1.4	81.4	16.4	0.8
Cases	0	0	0.5	4.9	80	14.6	0.5
Cases	0	0	0.8	3.9	79.7	15.6	0.8

DISTRIBUTION OF CLINICAL FEATURES AMONG CASES:

Group	Age Group of children	Sex	Consanginous Marriage	Family H/O	Neurological Signs	Dysmorphic Features	Ophthalmological Signs	Radiological Findings	Other Features
Cases	Paediatric	Female	Yes	No	Yes	No	No	Yes	No
Cases	Paediatric	Female	No	No	Yes	No	No	No	No
Cases	Paediatric	Male	Yes	No	Yes	No	No	No	No
Cases	Paediatric	Male	No	No	Yes	No	No	No	No
Cases	Paediatric	Female	Yes	No	Yes	No	No	No	No
Cases	Paediatric	Male	Yes	No	Yes	No	No	No	No
Cases	Paediatric	Female	No	Yes	Yes	No	No	No	Yes
Cases	Paediatric	Female	No	No	Yes	No	Yes	No	No
Cases	Paediatric	Male	Yes	No	Yes	No	No	Yes	No
Cases	Paediatric	Male	No	No	Yes	Yes	Yes	No	No
Cases	Paediatric	Female	No	Yes	Yes	Yes	No	Yes	No
Cases	Paediatric	Male	No	No	Yes	Yes	No	Yes	No
Cases	Paediatric	Male	Yes	No	Yes	Yes	Yes	Yes	No
Cases	Paediatric	Female	Yes	Yes	Yes	No	Yes	No	No
Cases	Paediatric	male	Yes	No	Yes	No	No	Yes	No
Cases	Paediatric	Male	No	No	Yes	Yes	No	Yes	No
Cases	Paediatric	Female	No	No	Yes	Yes	Yes	Yes	No
Cases	Paediatric	Male	No	Yes	Yes	No	Yes	No	No
Cases	Paediatric	Male	Yes	No	Yes	Yes	Yes	No	No
Cases	Paediatric	Male	Yes	No	Yes	No	No	No	No
Cases	Paediatric	Male	Yes	No	Yes	Yes	No	Yes	No
Cases	Paediatric	Female	No	Yes	Yes	No	Yes	No	No
Cases	Paediatric	Female	No	Yes	Yes	Yes	No	No	No
Cases	Paediatric	Male	No	Yes	Yes	Yes	No	No	No
Cases	Paediatric	Male	No	Yes	Yes	Yes	No	No	No
Cases	Paediatric	Male	Yes	No	Yes	No	No	Yes	No
Cases	Paediatric	Male	No	No	Yes	No	No	Yes	No
Cases	Paediatric	Male	No	No	Yes	No	Yes	No	No
Cases	Paediatric	Male	Yes	No	Yes	Yes	No	No	No
Cases	Paediatric	Male	No	Yes	Yes	Yes	Yes	No	No

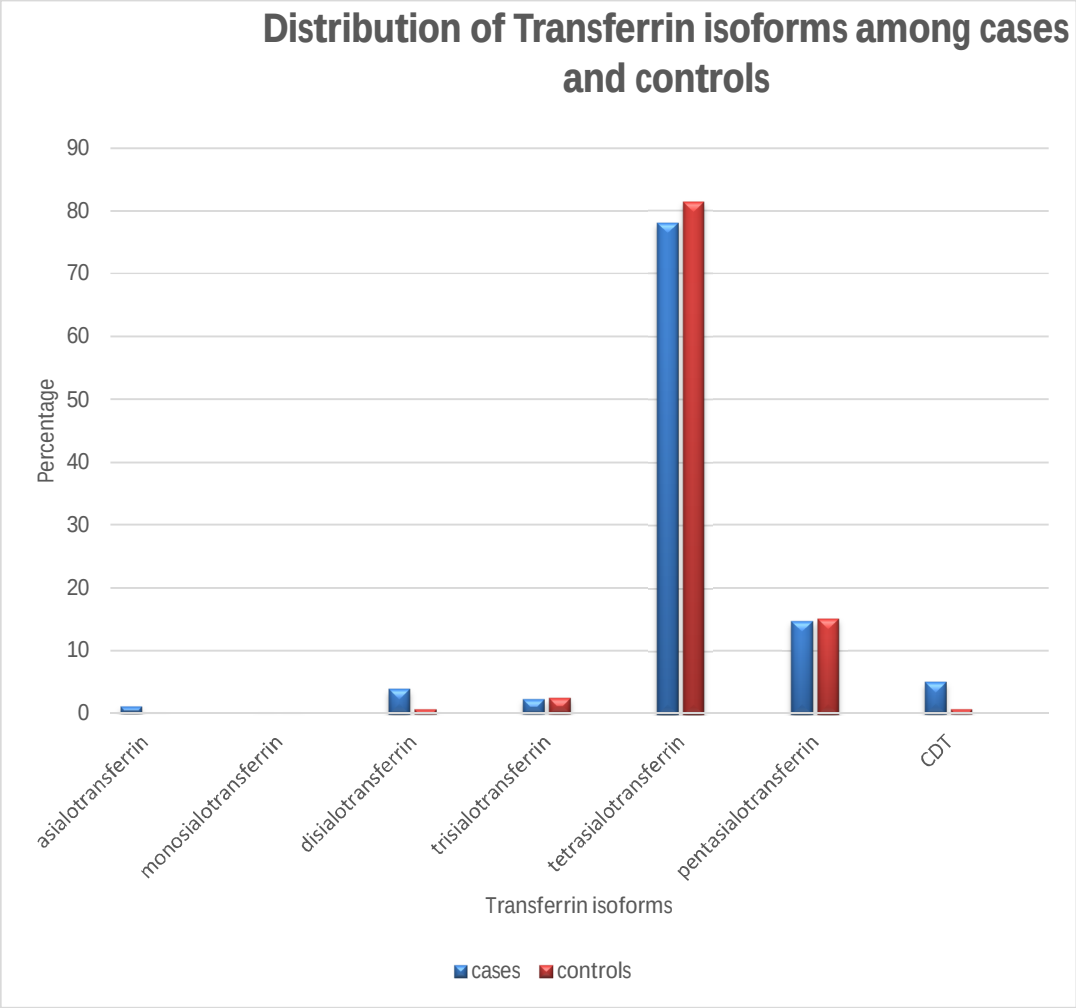
RESULTS

❖ **Distribution of transferrin isoforms among cases and control:**

TABLE 1:

Transferrin isoforms	Group	N	Mean (%)	Std. Deviation	Std. Error Mean
Asialotransferrin	Cases	30	1.0	3.24	.59
	Control	30	0	0	0
Monosialotransferrin	Cases	30	.01	.06	.01
	Control	30	0	0	0
Disialotransferrin	Cases	30	3.93	12.39	2.26
	Control	30	.75	.23	.04
Trisialotransferrin	Cases	30	2.25	1.20	.21
	Control	30	2.43	.83	.15
Tetrasialotransferrin	Cases	30	78.11	13.79	2.51
	Control	30	81.44	2.06	.37
Pentasialotransferrin	Cases	30	14.65	3.14	.57
	Control	30	15.09	2.09	.38
CDT	Cases	30	4.96	15.44	2.82
	Control	30	.75	.23	.04

Figure 13: Comparision of transferrin isoforms among the cases and controls



The above figure illustrates that the Asialo, Disialo and CDT isoforms are higher in the cases group when compared to the control group.

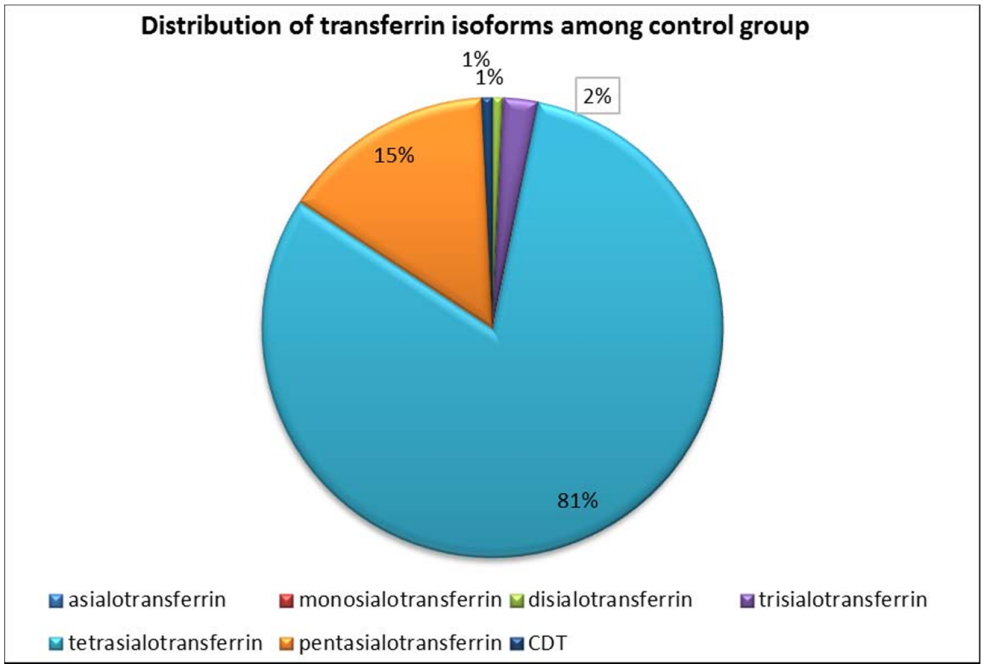
❖ **Distribution of transferrin isoforms among controls:**

TABLE 2:

Transferrin	N	Minimum (%)	Maximum (%)	Mean (%)	Std. Deviation
Asialo	30	0	0	0	0
Monosialo	30	0	0	0	0
Disialo	30	0.4	1.4	0.75	0.23
Trisialo	30	0.9	4.0	2.43	0.83
Tetrasialo	30	74.7	85.0	81.44	2.06
Pentasialo	30	11.2	20.7	15.09	2.09
CDT	30	0.4	1.4	0.75	0.23

Thus the mean (SD) of tranferrin isoforms among the controls were estimated and found to be Asialo transferrin-0 (0); monosialo transferrin- 0(0); disialo transferrin-0.75% (0.2) ; trisialotransferrin- 2.4% (0.8) ; tetrasialotransferrin- 81.4 % (2) ; pentasialotransferrin- 15.09% (2) and CDT-0.75% (0.2).

Figure 14: Distribution of transferrin isoforms among control group



Asialotransferrin and monosiaotransferrin was found to be absent in the control group.Distribution of disialotransferrin was 1%, trisialotransferrin was 2%, tetrasialotransferrin was 81% and pentasialotransferrin was 15% among the control group.

❖ **Statistical significance in the distribution of transferrin isoforms between adult and pediatric age group in controls:**

Among the control group 11 were pediatric samples and 19 were adult samples. Transferrin isoform distribution between the adult and pediatric age group was studied in the control group

TABLE 3:

Age Group of controls	Frequency	Percent
Peadiatric	11	36.7
Adult	19	63.3
Total	30	100.0

TABLE 4: Independent sample T test

Transferrin	Age Group of children	N	Mean (%)	Std. Deviation	P value
Asialo	Peadiatric	11	0	0(a)	-
	Adult	19	0	0(a)	
Monosialo	Peadiatric	11	0	0(a)	-
	Adult	19	0	0(a)	
Disialo	Peadiatric	11	0.78	0.16	0.578
	Adult	19	0.73	0.26	
Trisialo	Peadiatric	11	2.67	0.91	0.236
	Adult	19	2.29	0.76	
Tetrasialo	Peadiatric	11	80.58	3.02	0.082
	Adult	19	81.94	1.03	
Pentasialo	Peadiatric	11	15.96	2.73	0.084
	Adult	19	14.58	1.48	
CDT	Peadiatric	11	0.78	0.16	0.578
	Adult	19	0.73	0.26	

(a) t cannot be computed because the standard deviations of both are zero

It was found that no statistical significant difference occurs in the distribution of transferrin isoforms between the paediatric and adult population.

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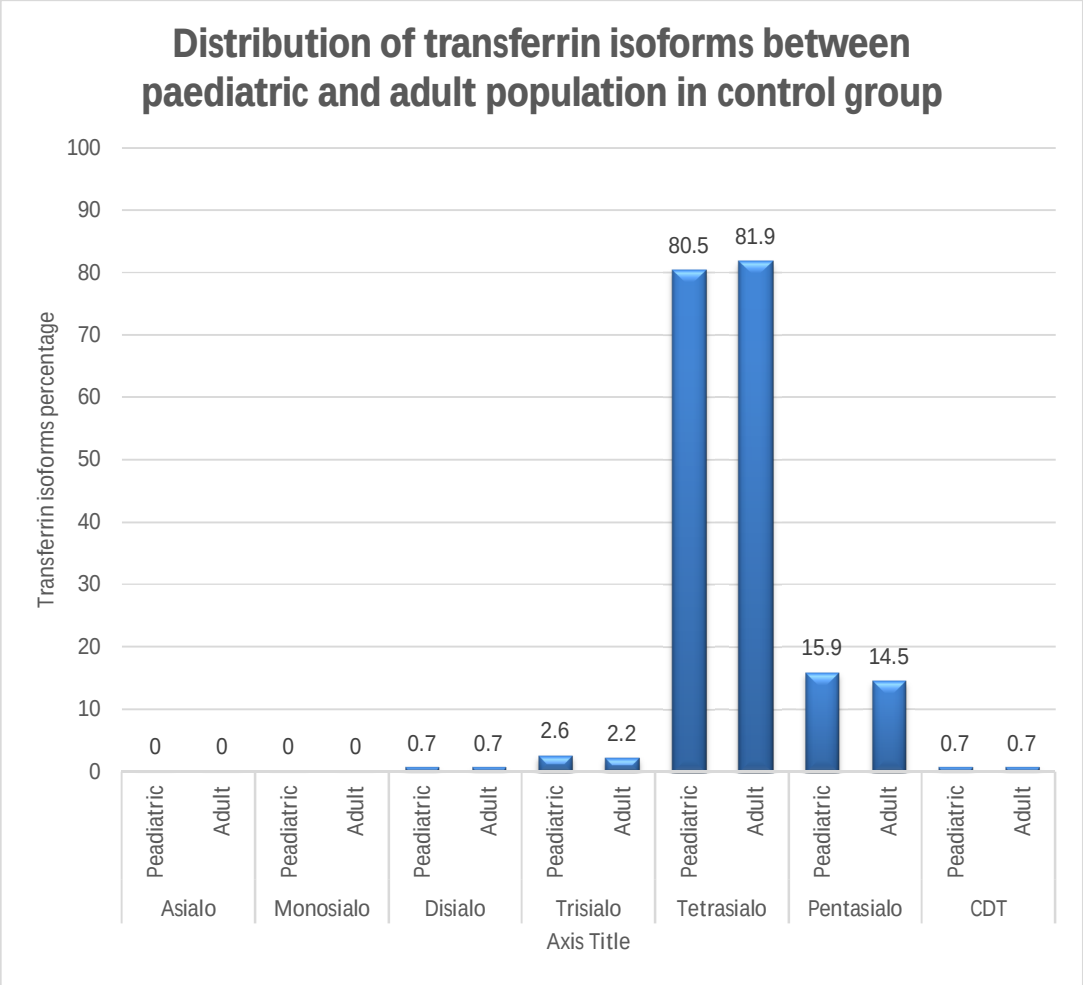
Figure 15: Distribution of transferrin isoforms between paediatric and adult population in control group



❖ **Statistical significance in the distribution of transferrin isoforms between males and females in controls:**

Among the control group 16 were males and 14 were females. Transferrin isoform distribution between the males and females was studied in the control group

TABLE 5:

Sex of controls	Frequency	Percent
Male	16	53.3
Female	14	46.7
Total	30	100.0

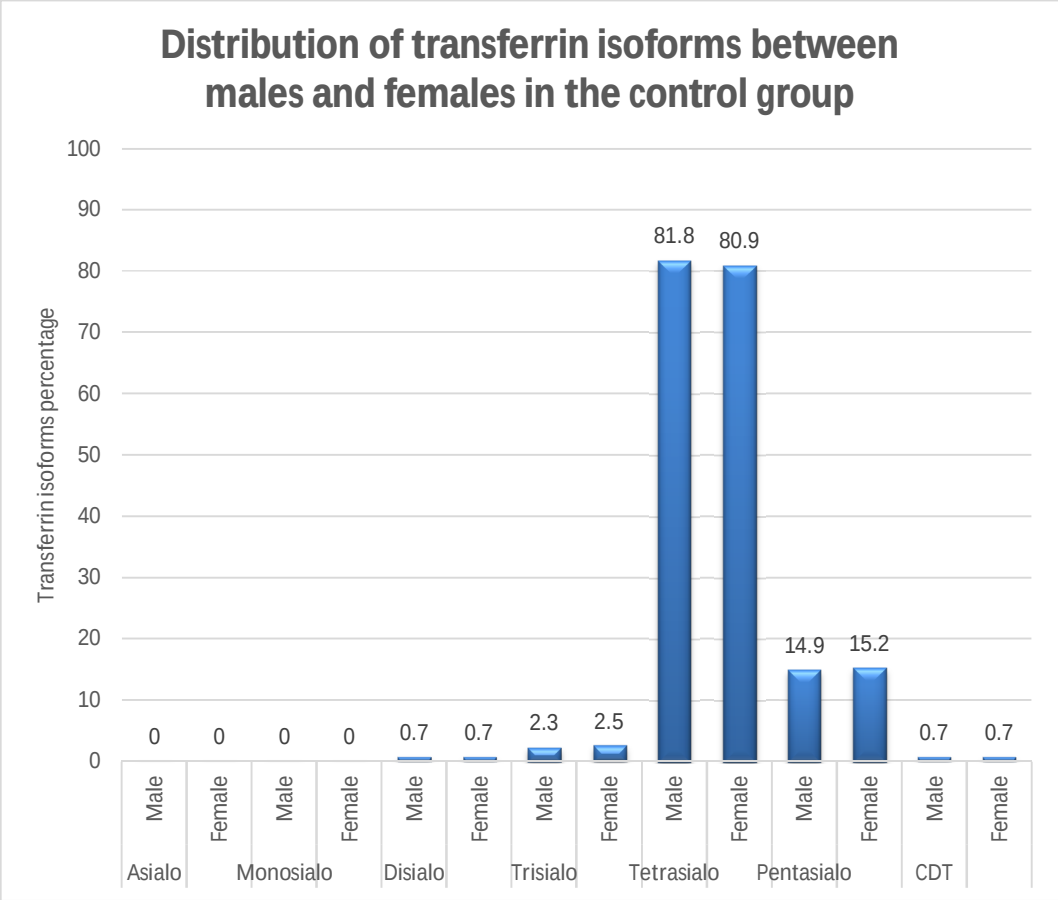
TABLE 6: Independent sample T test

Transferrin	Sex	N	Mean (%)	Std. Deviation	P value
Asialo	Male	16	0	0(a)	-
	Female	14	0	0(a)	
Monosialo	Male	16	0	0(a)	-
	Female	14	0	0(a)	
Disialo	Male	16	0.74	0.21	0.878
	Female	14	0.75	0.25	
Trisialo	Male	16	2.36	0.79	0.657
	Female	14	2.50	0.89	
Tetrasialo	Male	16	81.83	1.76	0.660
	Female	14	80.99	2.34	
Pentasialo	Male	16	14.98	1.88	0.760
	Female	14	15.22	2.38	
CDT	Male	16	0.74	0.21	0.878
	Female	14	0.75	0.25	

(a) t cannot be computed because the standard deviations of both are zero

It was found that no statistical significant difference occurs in the distribution of transferrin isoforms between the male and female population in the control group.

Figure 16: Distribution of transferrin isoforms between males and females in the control group



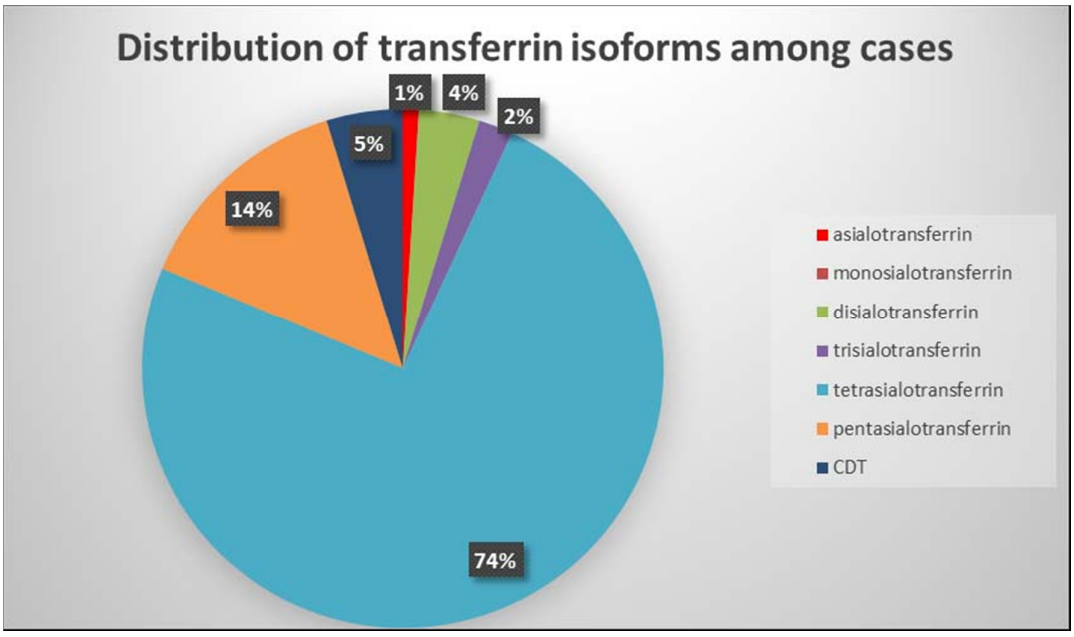
❖ **Distribution of transferrin isoforms in cases:**

TABLE 7:

Transferrin	N	Minimum (%)	Maximum (%)	Mean (%)	Std. Deviation
Asialo	30	.0	13.8	1.03	3.24
Monosialo	30	.0	0.3	0.01	0.06
Disialo	30	.3	52.6	3.93	12.39
Trisialo	30	.2	4.9	2.25	1.24
Tetrasialo	30	24.4	86.2	78.11	13.79
Pentasialo	30	6.2	23.2	14.65	3.14
CDT	30	.3	66.4	4.96	15.44

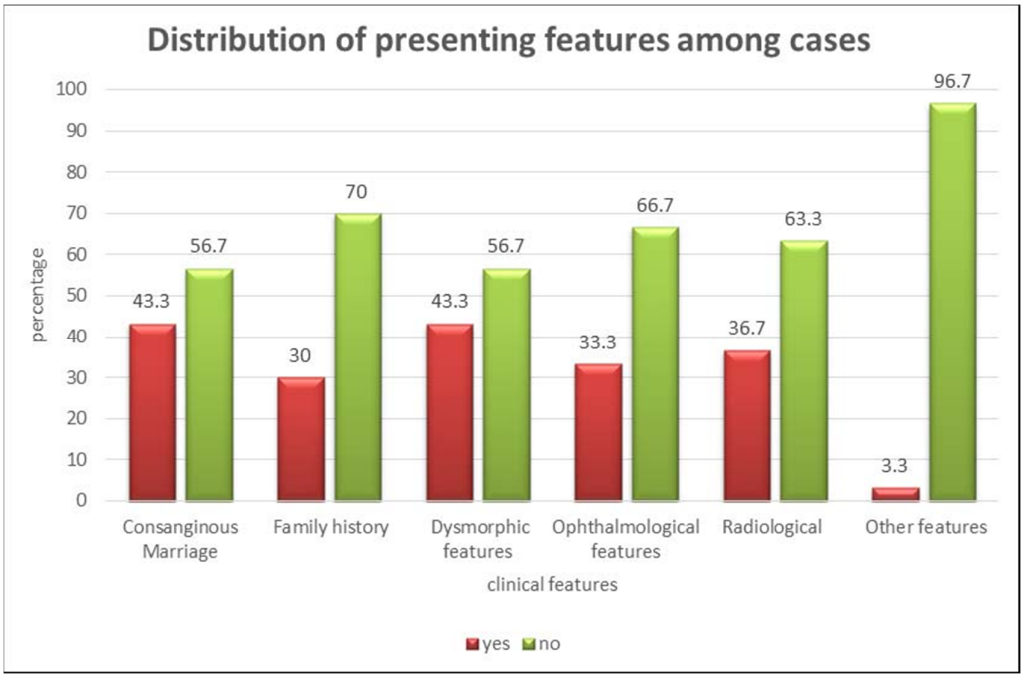
Thus the mean (SD) of tranferrin isoforms among the cases were estimated and found to be Asialo transferrin-1.03% (3.24); monosialo transferrin- 0.01% (0.06); disialo transferrin-3.93% (12.39) ; trisialotransferrin- 2.25% (1.24) ; tetrasialotransferrin- 78.11 % (13.79) ; pentasialotransferrin- 14.65 % (3.14) and CDT-4.96% (15.44)

Figure 17: Distribution of transferrin isoforms among cases



- **Distribution of transferrin isoforms among various presenting featrures in cases**

Figure 18: Distribution of presenting features among cases



Consanguinous marriage

The 30 cases were divided into two groups i.e. the group with H/O consanguinous marriage in parents and the group with no H/O consanguinous marriage in parents.

Among the 30 pediatric samples obtained for cases, 13 had consanguinity and 17 were non non consanginous.

TABLE 8 :

Consanguinous Marriage	Frequency	Percent
Yes	13	43.3
No	17	56.7
Total	30	100.0

The figure illustrates that the percentage of asialo transferrin, disialotransferrin and CDT are increased in the group with history of consanginous marriage when compared to the group with no history of consanginous marriage

Figure 19: frequency of consanguinity and nonconsanguinity among cases

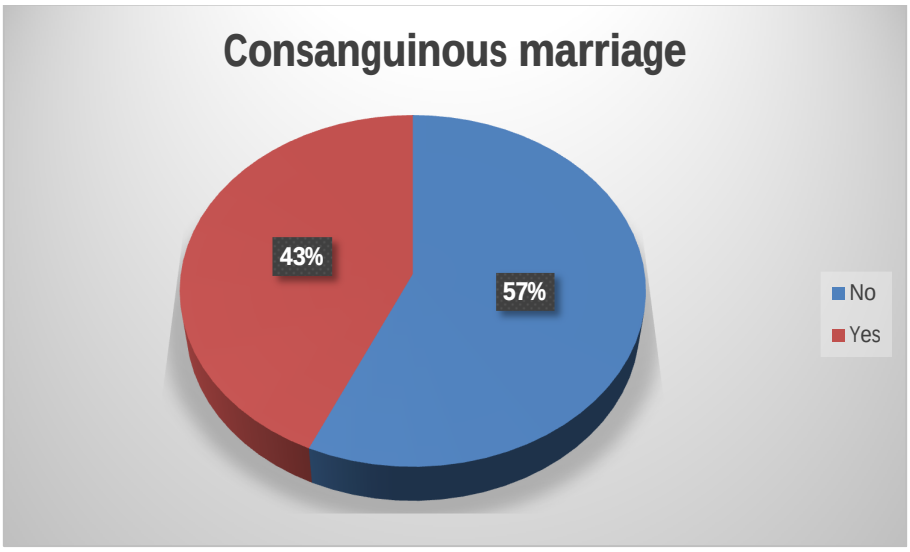


Figure 20: Distribution of transferrin isoforms between the group with history of consanguinous marriage and history of non consanguinous marriage in parents.

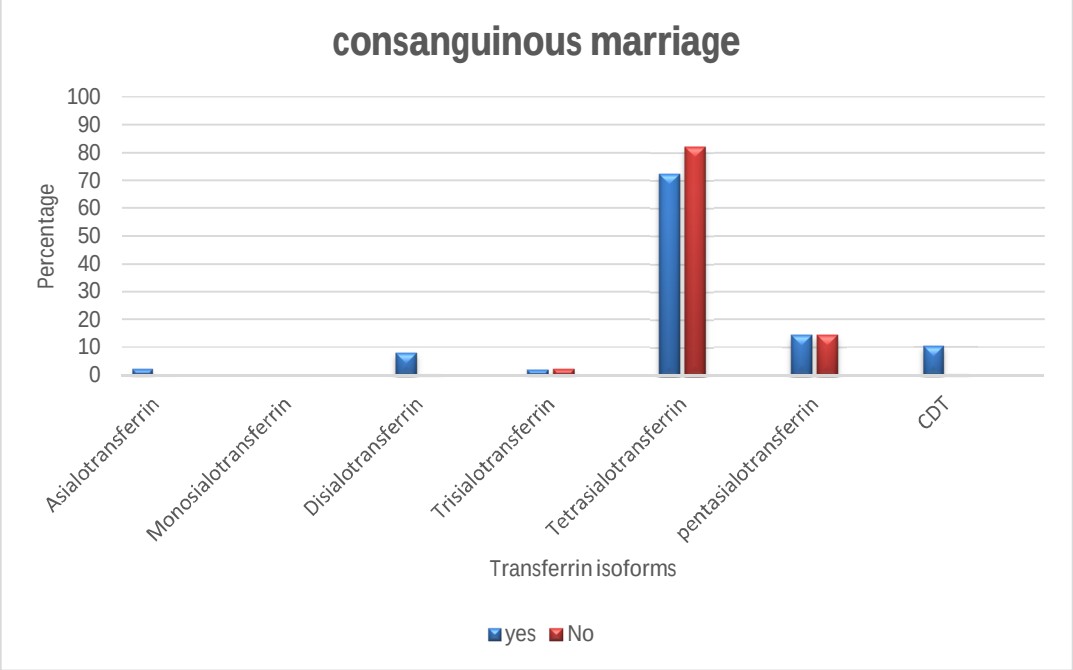


TABLE 9: Independent sample T test

	Consanginous	N	Mean (%)	Std. Deviation	Std. Error Mean	P value
Asialotransferrin	Yes	13	2.35	4.70	1.30	0.050*
	No	17	.02	0.09	0.02	
Monosialotransferrin	Yes	13	.03	0.09	0.02	0.108
	No	17	0	0	0	
Disialotransferrin	Yes	13	8.26	18.30	5.07	0.094
	No	17	0.61	0.19	0.04	
Trisialotransferrin	Yes	13	2.07	1.27	0.35	0.483
	No	17	2.39	1.15	0.28	
Tetrasialotransferrin	Yes	13	72.63	19.93	5.52	0.056
	No	17	82.29	1.92	0.46	
Pentasialotransferrin	Yes	13	14.62	4.30	1.19	0.964
	No	17	14.67	1.99	0.48	
CDT	Yes	13	10.62	22.70	6.29	0.079
	No	17	0.63	0.18	0.04	

Transferrin isoform distribution was studied between the two groups. It was found that statistical significant differrence occurs in the distribution of asialo transferrin between the two groups.

Family history:

The 30 pediatric samples obtained for cases were divided into two groups i.e. the group with similar history in the family and the group with no similar family history.

TABLE 10:

Family H/O		Frequency	Percent
Valid	No	21	70.0
	Yes	9	30.0
	Total	30	100.0

TABLE 11: Independent sample T test

	familyhistory	N	Mean (%)	Std. Deviation	Std. Error Mean	P value
asialotransferrin	Yes	9	0	0	0	0.261
	No	21	1.47	3.82	0.83	
monosialotransferrin	Yes	9	0	0	0	0.365
	No	21	0.02	0.07	0.01	
disialotransferrin	Yes	9	0.64	0.18	0.06	0.351
	No	21	5.33	14.68	3.20	
trisialotransferrin	Yes	9	2.18	1.36	0.45	0.844
	No	21	2.28	1.15	0.25	
tetrasialotransferrin	Yes	9	81.35	2.74	0.91	0.408
	No	21	76.71	16.30	3.55	
pentasialotransferrin	Yes	9	15.81	3.25	1.08	0.191
	No	21	14.15	3.03	0.66	
CDT	Yes	9	0.64	0.18	0.06	0.325
	No	21	6.81	18.27	3.98s	

Transferrin isoform distribution was studied between the two groups. It was found that no statistical significant differrence occurs in the distribution of transferrin isoforms between the two groups.

Figure 21: frequency of similar family history and no similar family history in the cases

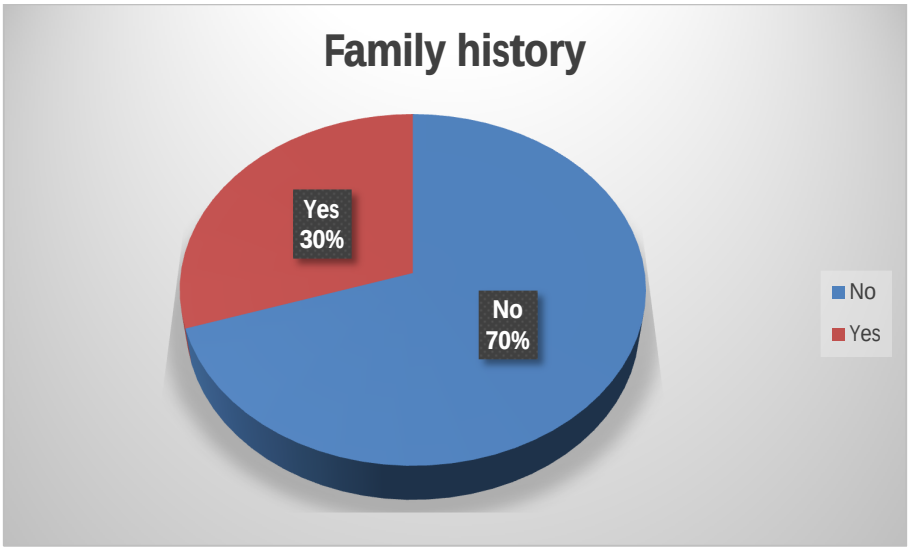


Figure 22: Distribution of transferrin isoforms between cases with similar family history and cases with no similar family history

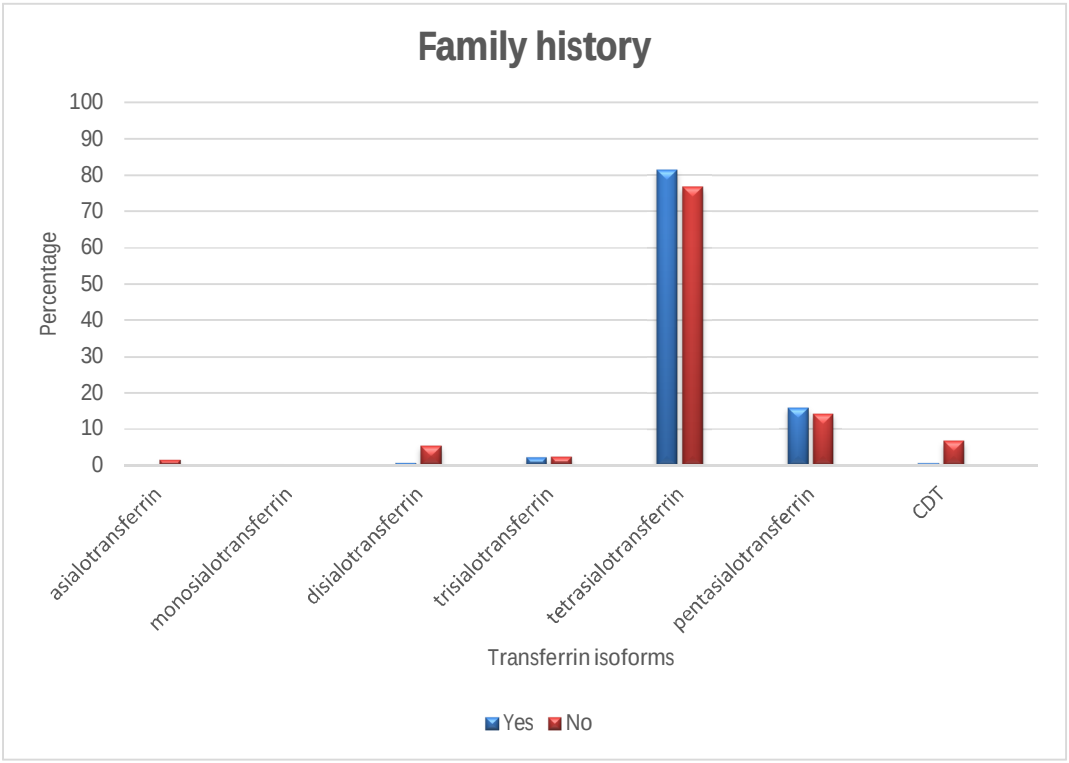


TABLE 12: Neurological Signs

Neurological Signs		Frequency	Percent
	Yes	30	100.0

Of the 30 samples obtained for screening, all the patients presented with variety of neurological manifestations among which seizures, mental retardation, developmental delay and hypotonia were the most common clinical symptoms.

Dysmorphic Features

The 30 pediatric samples obtained for cases were divided into two groups i.e. the group with dysmorphic features and the group with no dysmorphic features.

TABLE 13:

Dysmorphic Features		Frequency	Percent
	No	17	56.7
	Yes	13	43.3
	Total	30	100.0

TABLE 14: Independent sample T test

	Dysmorphic	N	Mean (%)	Std. Deviation	Std. Error Mean	P value
Asialotransferrin	Yes	13	0.03	0.11	0.03	0.142
	No	17	1.80	4.20	1.019	
Monosialotransferrin	Yes	13	0	0	0	0.224
	No	17	0.02	0.08	0.02	
Disialotransferrin	Yes	13	0.60	0.22	0.06	0.204
	No	17	6.47	16.19	3.92	
Trisialotransferrin	Yes	13	2.47	1.34	0.37	0.389
	No	17	2.08	1.08	0.26	
Tetrasialotransferrin	Yes	13	81.68	2.01	0.55	0.220
	No	17	75.37	17.98	4.36	
pentasialotransferrin	Yes	13	15.20	2.01	0.55	0.414
	No	17	14.23	3.79	0.92	
CDT	Yes	13	0.63	0.22	0.06	0.185
	No	17	8.27	20.14	4.88	

Transferrin isoform distribution was studied between the two groups.

Transferrin isoform distribution was studied between the two groups. It was found that no statistical significant differrence occurs in the distribution of transferrin isoforms between the two groups.

Figure 23: frequency of dysmorphic features and no dysmorphic features in cases

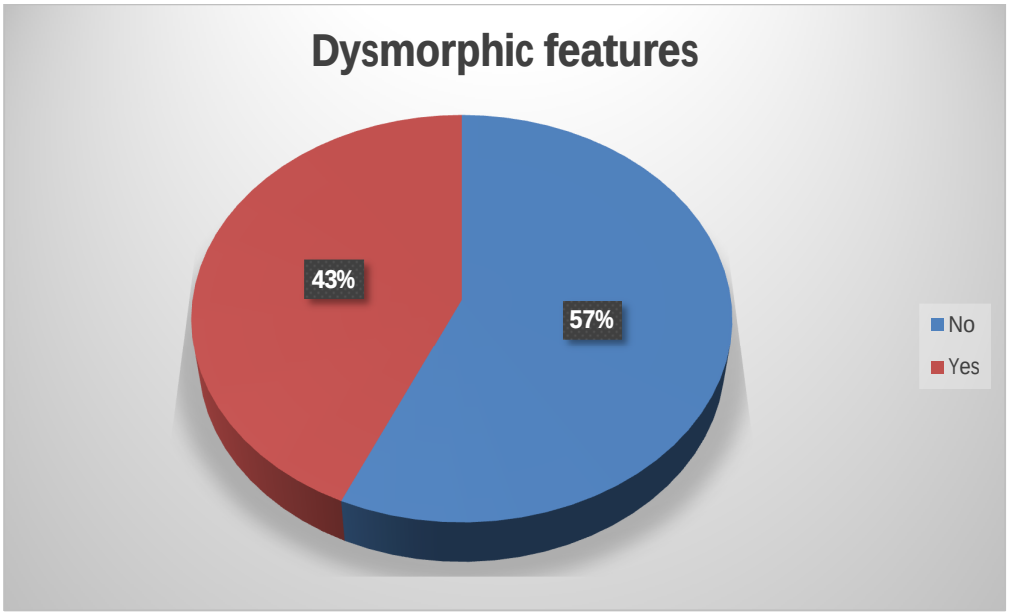
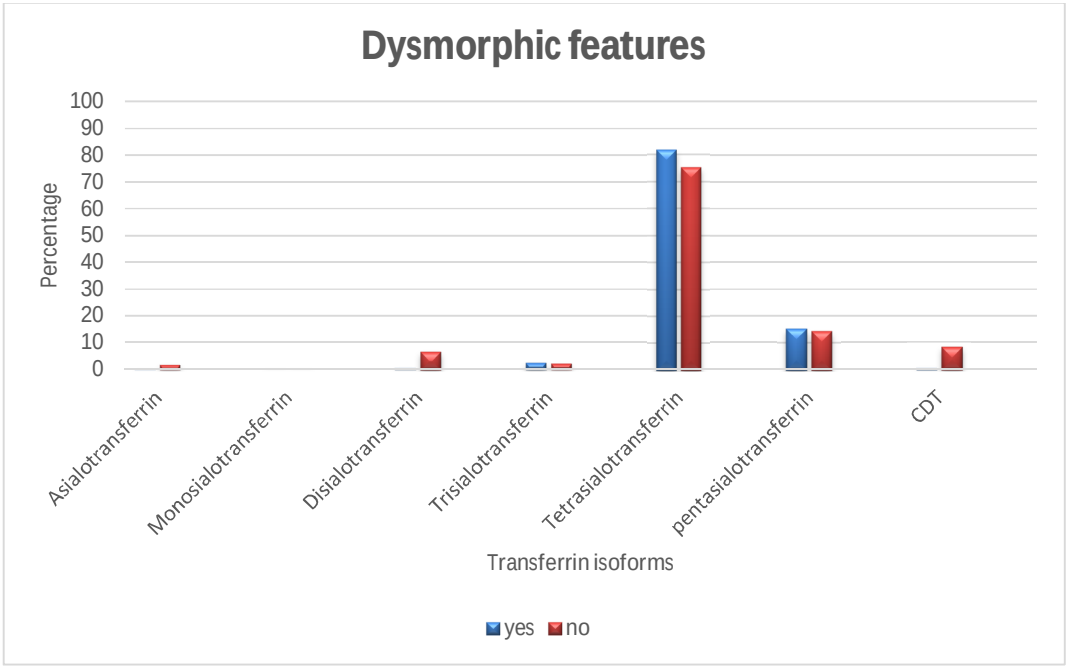


Figure 24: Distribution of transferrin isoforms between the group with dysmorphic features and the group with no dysmorphic features in cases.



Ophthalmological Signs:

The 30 pediatric samples obtained for cases were divided into two groups i.e. the group with ophthalmological signs and the group with no ophthalmological signs.

TABLE 15:

Ophthalmological Signs		Frequency	Percent
	No	20	66.7
	Yes	10	33.3
	Total	30	100.0

TABLE 16: Independent sample T test

	ophthal	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**	**P value**
Asialotransferrin	Yes	10	0	0	0	0.224
	No	20	1.55	3.90	0.87	
Monosialotransferrin	Yes	10	0	0	0	0.328
	No	20	0.02	0.07	0.01	
Disialotransferrin	Yes	10	0.73	0.18	0.05	0.326
	No	20	5.53	15.04	3.36	
Trisialotransferrin	Yes	10	2.33	1.28	0.40	0.818
	No	20	2.22	1.19	0.26	
Tetrasialotransferrin	Yes	10	81.87	2.74	0.86	0.299
	No	20	76.23	16.59	3.71	
pentasialotransferrin	Yes	10	15.07	3.50	1.10	0.616
	No	20	14.44	3.01	0.67	
CDT	Yes	10	0.73	0.18	0.05	0.297
	No	20	7.0800	18.71100	4.18391	

Transferrin isoform distribution was studied between the two groups. It was found that no statistical significant differrence occurs in the distribution of transferrin isoforms between the two groups.

Figure 25: frequency of ophthalmological signs and no ophthalmological signs in cases

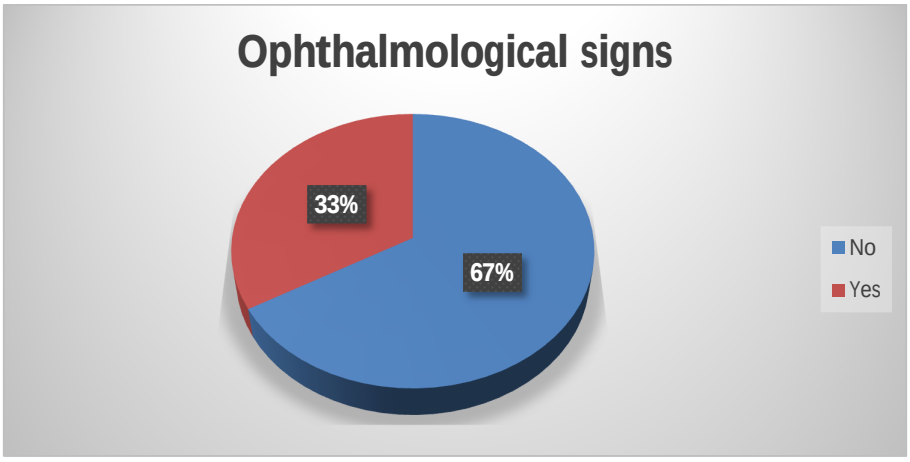
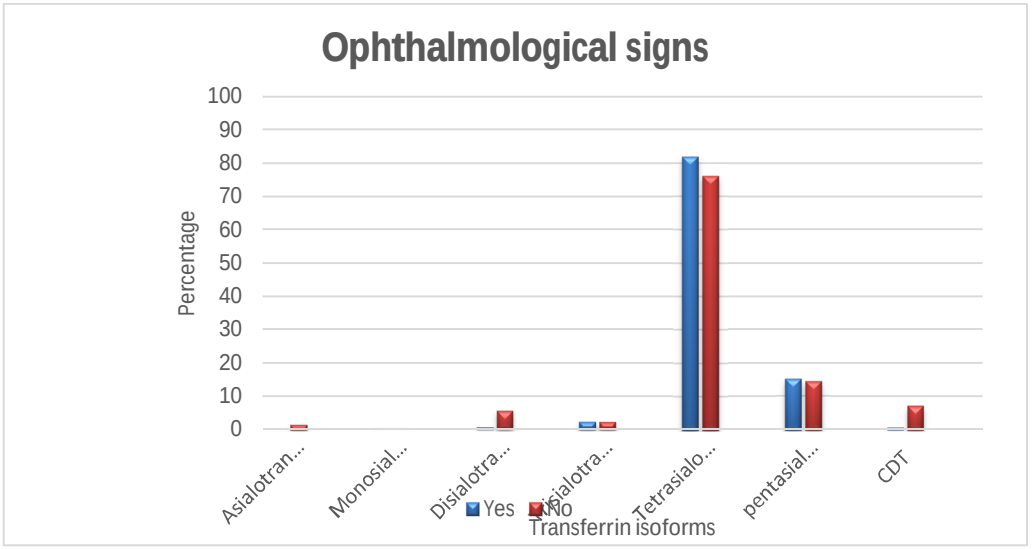


Figure 26: Distribution of transferrin isoforms between the group with ophthalmological and the group with no ophthalmological signs



Radiological Findings

The 30 pediatric samples obtained for cases were divided into two groups i.e. the group with radiological finding and the group with no radiological finding.

TABLE 17:

Radiological Findings		Frequency	Percent
	No	19	63.3
	Yes	11	36.7
	Total	30	100.0

TABLE 18: Independent sample T test

	Radiological features	**N**	**Mean (%)**	**Std. Deviation**	**Std. Error Mean**	**P value**
Asialotransferrin	Yes	11	2.81	5.00	1.51	0.019*
	No	19	0	0	0	
Monosialotransferrin	Yes	11	0.04	0.10	0.03	0.063
	No	19	0	0	0	
Disialotransferrin	Yes	11	9.52	19.76	5.95	0.058
	No	19	0.68	0.22	0.05	
Trisialotransferrin	Yes	11	2.33	0.90	0.27	0.787
	No	19	2.21	1.36	0.31	
Tetrasialotransferrin	Yes	11	72.02	21.86	6.59	0.065
	No	19	81.63	2.26	0.51	
pentasialotransferrin	Yes	11	13.24	3.45	1.04	0.060
	No	19	15.46	2.70	0.62	
CDT	Yes	11	12.34	24.44	7.36	0.044*
	No	19	0.68	0.22	0.05	

Transferrin isoform distribution was studied between the two groups. It was found that statistical significant differrence occurs in the distribution of asialotransferrin isoform and CDT between the two groups.

Figure 27: frequency of radiological findings and no radiological findings in cases

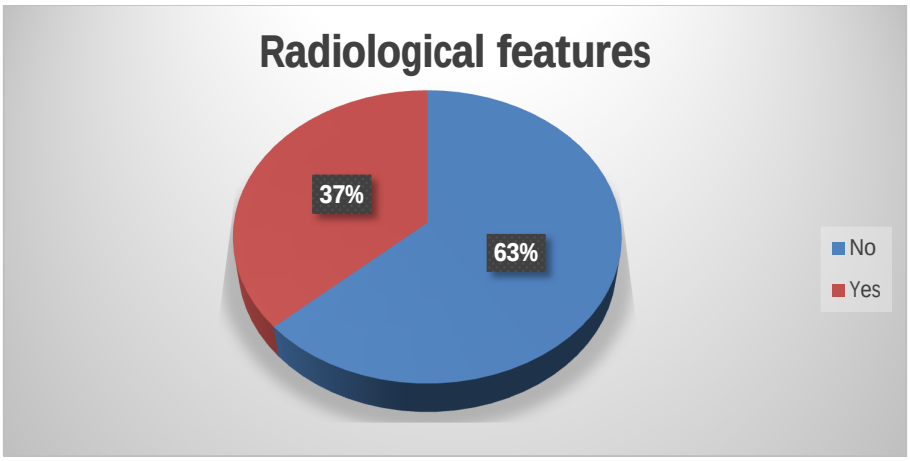
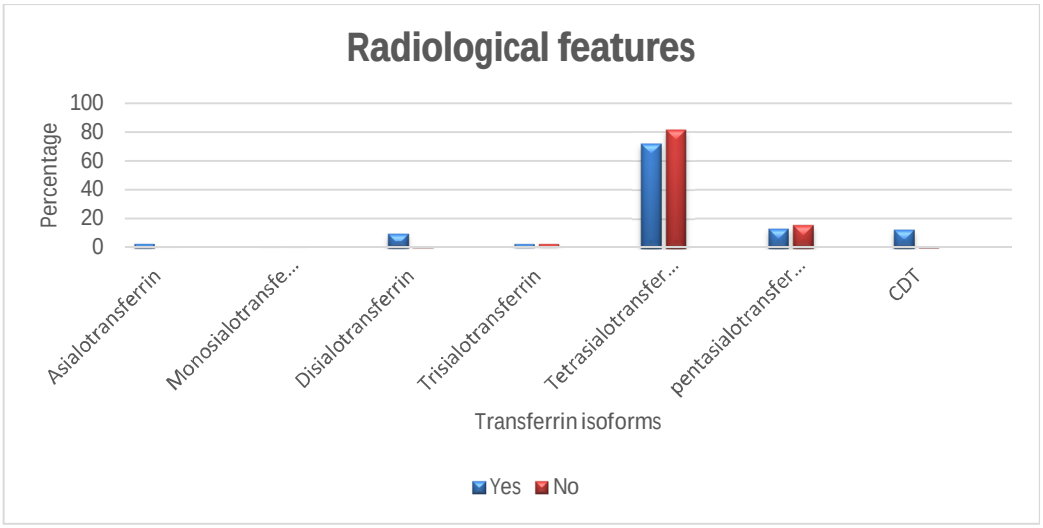


Figure 28: Distribution of transferrin isoforms between the group with radiological findings and the group with no radiological findings in the cases group



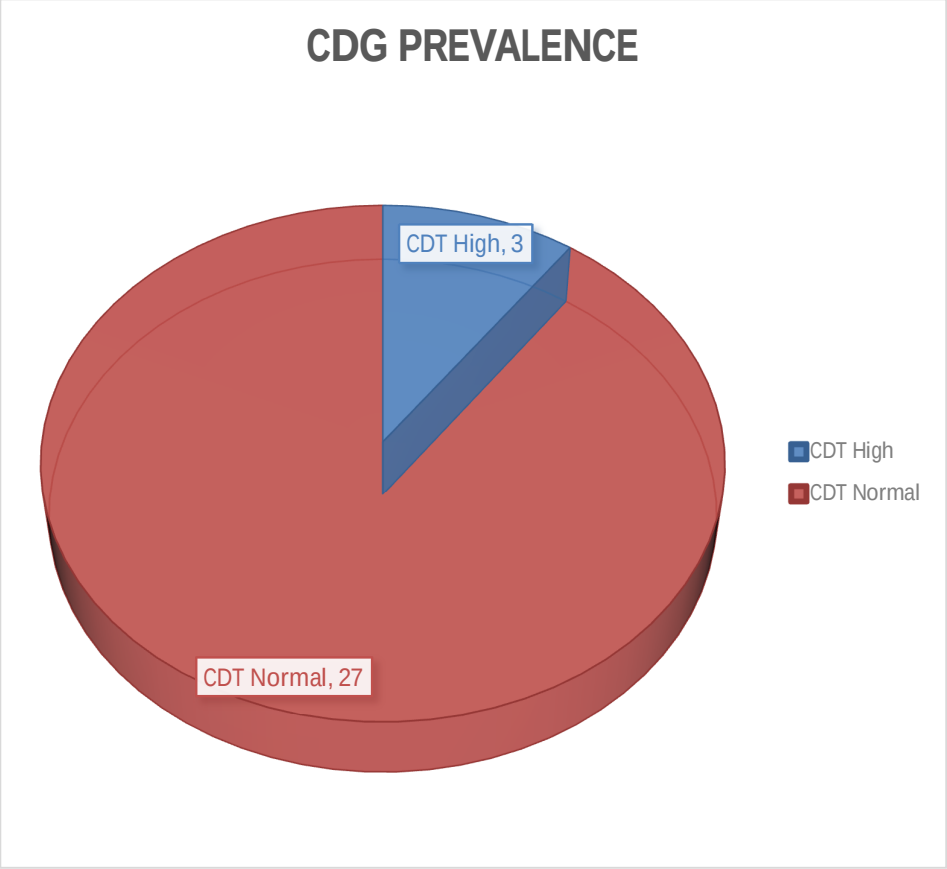
7. Prevalence of CDT positive samples among cases:

TABLE 19: (chi square test)

CDT	No of cases	Percent %	P value
High	3	10%	<0.001**
Normal	27	90 %	
Total	30	100	

The findings shows that among 30 cases evaluated for screening, three cases has showed increased carbohydrate deficient transferrin isoform (asialo and disialo transferrin). Thus in this study we have detected a prevalence of 10% CDG among the 30 patients which is statistically highly significant.

Figure 29: Prevalence of CDG among cases



Discussion

DISCUSSION

Glycoproteins play multiple significant roles in cell biology. Defect in the glycosylation pattern of glycoproteins affect their function and leads to a group of disease known as congenital disorders of glycosylation. The basic defect in this disorder is a mutant gene which encodes the enzymes responsible for glycosylation of glycoproteins. In the study on CDG by Jaeken in 2001 a prevalence of 1 in 20,000 worldwide[101] has been reported.In our country no formal study on prevalence of CDG has been undertaken to our knowledge.There are also no reports about any study or case report of CDG case among Indians in reputed journals.Hence we are unable to compare population prevalence of CDG.

CDG presents with a broad clinical features affecting multiple organ systems in the body (especially the nervous system, muscles, intestines & immune system). Moreover the range of severity of this disorder is also very wide i.e. CDG can be very mild so that the patient is asymptomatic or it can also be fatal leading to death. There is also lack of awareness and access to the laboratory techniques available to evaluate this disorder. All these factors make it difficult for the clinicians in diagnosing this disorder.

Though CDG is a disorder with broad clinical presentation, neurological manifestations such as seizure disorder, delayed milestones and mental retardation are the most common presentations. CDG being an autosomal recessive condition which runs in families, consanguinous marriage remains to be a significant risk factor for the inheritance of this disorder.

Since consanguinous marriage and neurological symptoms like seizures are more prevalent in our population, this study aimed to screen for CDG in our population using capillary zone electrophoresis of serum transferrin. Although glycosylation pattern of other glycoproteins can be studied, transferrin is the preferred glycoprotein because it is the most abundant glycoprotein in serum and the glycosylation of this glycoprotein does not change with age when compared to other glycoproteins.

The gold standard technique for the diagnosis of CDG is isoelectric focussing of serum transferrin which is a cumbersome manual technique. Therefore we have chosen capillary zone electrophoresis of serum transferrin which is an automated precise technique to screen this disorder.

In this study control samples were obtained from apparently healthy normal individuals of varying sexes and age groups after ruling out the history of alcoholism since chronic alcoholism interferes with the CDT assay.

Samples for cases were obtained from pediatric patients who were clinically suspected to suffer from CDG. Other differential diagnosis such as inborn error of aminoacid metabolism, fatty acid oxidation defects,structural anomalies of brain were excluded by the possible investigations such as urine metabolic screening, tandem mass spectrometry analysis of dried blood spot for detection of disorders of amino acids/fattyacid oxidation defects/ organic acidemias ,CT brain, ophthalmological and ENT evaluation. In such patients

whose diagnosis still remained inconclusive but the clinical symptoms were suggestive of CDG were screened by CZE of serum transferrin to find the prevalence of the disease.

Out of 30 cases screened for CDG using CZE three cases showed abnormal transferrin glycosylation pattern.The pattern is similar to the pattern “A” reported by Fabiene (most frequent pattern in CDG) which is consistent with the complete loss of glycans. Thus the prevalence of CDG is 10% among 30 cases.

Transferrin glycosylation pattern of 30 controls were evaluated. Transferrin isoform distribution was compared between adult and pediatric age group & between males and females.It is found that there is no statistical significance occurs in the distribution of transferrin isoforms between the different age groups and sexes in the control group.

Based on clinical features the cases were divided into different groups and the transferrin glycoforms were compared between the groups.The groups are

1. Consangineous marriage Vs non consangineous marriage
2. Ophthalmological signs Vs no ophthalmological signs
3. Dysmorphic features Vs no dysmorphic features
4. Similar family history Vs no similar family history
5. Radiological findings Vs no radiological findings
6. Other clinical features Vs no other clinical features

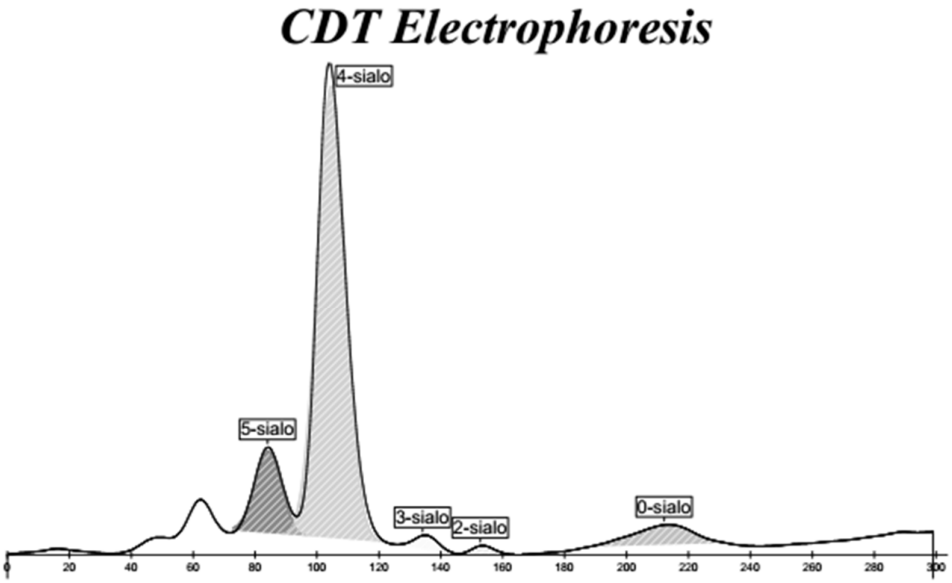
It was found that there is statistically significant increase of asialo transferrin isoform in the consangineous marriage group when compared to the non consanginous marriage group. As asialotransferrin contributes to the measure of CDT, this finding concludes that consanginous marriage is a significant risk factor for CDG.

Simillarly a significant statistical difference is found in the distribution of asialotransferrin isoforms and CDT in the group with positive radiological finding (cerebral atrophy) when compared to the group with no radiological finding. Therefore radiological evidence adds as supportive evidence in the diagnosis of CDG.

Other methods available for diagnosis of CDG:

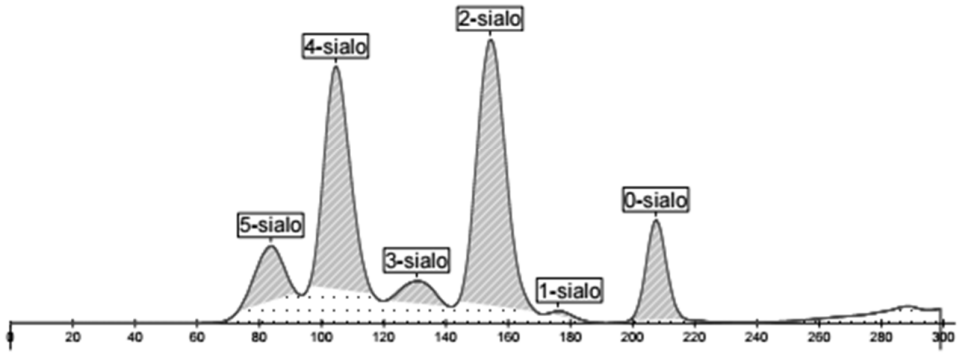
CDT Methods	Separation Principle	Glycoform Detection Method
IEF	Charge separation	i.Electrotransfer,immunoblot,detection with secondary antibody ii.Coomassie blue staining
CDTect	Charge separation	RIA of resolved fractions
%CDT TIA	Charge separation	Immunoturbidimetry of resolved fractions
CE	Charge separation	O.D. at 214nm
HPLC	Charge separation	O.D. at 640 nm
Electrospray mass spectrometry	Protein size	Summations of ions of a charged protein

Figure 30: Serum Transferrin electrophoresis pattern of 3 cases with elevated CDT in our study population:



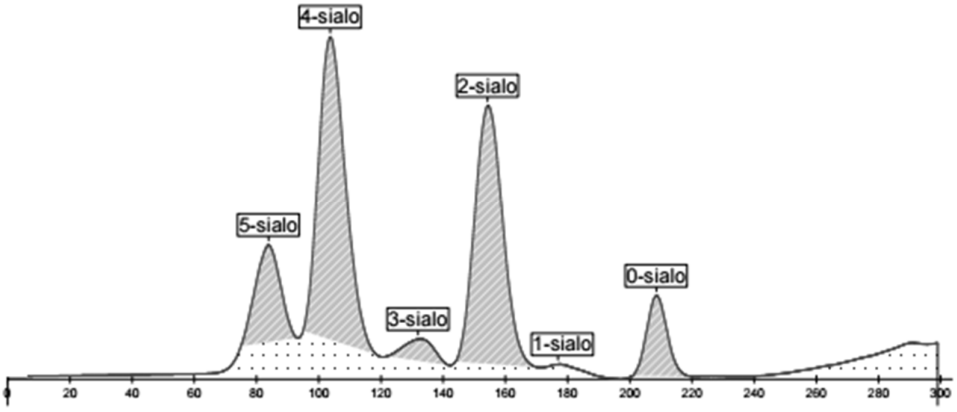
Transferrin isoforms	Percentage (%)	Normal range %
Asialo transferrin	6.6	<0.5
Monosialotransferrin	0.0	<0.9
Disialotransferrin	0.9	<2.5
Trisialotransferrin	1.7	4.5 – 9.0
Tetrasialotransferrin	77.8	64.8-80.0
Pentasialotransferrin	13	12.0-18.0
CDT	7.5	<1.3

CDT Electrophoresis



Transferrin isoforms	Percentage (%)	Normal range %
Asialo transferrin	10.2	<0.5
Monosialotransferrin	0.2	<0.9
Disialotransferrin	46.2	<2.5
Trisialotransferrin	2.4	4.5 – 9.0
Tetrasialotransferrin	31.9	64.8-80.0
Pentasialotransferrin	9.1	12.0-18.0
CDT	56.4	<1.3

CDT Electrophoresis



Transferrin isoforms	Percentage (%)	Normal range %
Asialo transferrin	13.8	<0.5
Monosialotransferrin	0.3	<0.9
Disialotransferrin	52.6	<2.5
Trisialotransferrin	2.7	4.5 – 9.0
Tetrasialotransferrin	24.4	64.8-80.0
Pentasialotransferrin	6.2	12.0-18.0
CDT	66.4	<1.3

Arndt states that “Increased fractions of trisialotransferrin in Arginosuccinate lyase deficiency/urea cycle disorder(prevalence 1 in 35,000) patients appear to interfere with CDT analysis by the %CDT TIA assay. This can give false-positive CDT results. %CDT TIA assay should no longer be used for CDT measurement without confirmatory analysis by HPLC or capillary electrophoresis” [102,103]

Study conducted by Quintana states that “measurement of transferrin isoforms and interpretation using method-specific reference values in HPLC and CZE may offer some advantages for the diagnosis of CDG as compared with the standard IEF procedure” [104]

Fabienne Parente states that “Capillary zone electrophoresis of serum transferrin is an alternaive technique which can be used to screen the patients suspected with CDG”[70].

Fabienne Parente in the journal of Clinica Chimica Acta november 2010 conducted a study to evaluate the CZE in screening CDG , two types of pattern were obtained from CDG confirmed cases in his study.They were pattern A and pattern B[70] . Pattern A showed a characteristic decrease of penta and tetra and an increase of di and asialotransferrins, consistent with a loss of complete glycans Pattern B which showed a characteristic decrease of penta and tetra & increase of trisialotransferrins consistent with structurally abnormal glycans.

But CZE techniqe has limitations such as interferences from hemoglobin, fibrinogen, transferrin polymorphisms, complement fraction C3b & monoclonal immunoglobulins.

Parente study states that “There is no statistically significant difference between the different age groups (0–5, 6–11, 12–15, 16–18, and >18y) or between sexes for transferrin isoforms. Reference intervals obtained at 95% confidence interval was 5-sialo: 12.9 to 32.4 4-sialo: 65.9 to 82.9, 3-sialo: 0.4 to 5.6, CDT (di, mono,asialo):0.0 to 1.3”[70]

Briones study states that “Congenital disorders of glycosylation (CDG) and mitochondrial diseases are multisystem disorders with clinical characteristics that may overlap. As the search for the primary defect in mitochondrial diseases is often unsuccessful, the pool of mitochondrial patients that remain without definite diagnosis might include CDG cases. Routine screening for CDG may avoid precocious invasive investigation” [105] .

Charge difference in transferrin may occur because of amino acid polymorphisms and completeness of iron saturation which can affect the electrophoretic pattern of capillary zone electrophoresis. Sample pretreatment with iron eliminates the bias due to charge differenc caused by incomplete iron saturation.But the interference due to aminoacid polymorphism cannot be avoided in capillary zone electrophoresis.

Thus the advantage in using MS is that [106]

Transferrin does not have to be saturated with iron before analysis of the specimen since only apotransferrin is detected and Amino acid sequence polymorphisms also does not interfere with the MS resolution.

Summary & Conclusion

SUMMARY AND CONCLUSION

Congenital disorder of glycosylation is an inborn error of metabolism due to defective glycosylation of glycoproteins/glycolipids.It is a multisystem disorder with wide range of clinical presentations thereby making the clinical diagnosis difficult. Hence the diagnosis of CDG is entirely based upon laboratory investigations.Since CDG is a disorder due to defective glycosylation of glycoproteins, glycosylation pattern of a glycoprotein should be evaluated to diagnose this disorder.Transferrin is a glycoprotein whose glycosylation pattern does not change with age when compared to other glycoproteins. Therefore isoelectric focussing of serum transferrin is a gold standard technique to diagnose this disorder.

CDG prevalence is undoubtedly underestimated in our population due to lack of awareness and availability of automated, quick access diagnostic techniques.

Since about 2 % of human genome encodes proteins for Glycosylation it is likely that more disorders and more affected individuals will be found as awareness and diagnostic testing methods improve.

This study consisted of two groups. Control group with 30 samples of apparently healthy individuals and cases group with 30 patients who were clinically suspected to have congenital disorder of glycosylation. Other differential diagnosis of their symptoms were excluded using possible

investigations such as Urine metabolic screening, Tandem mass spectrometry for detection of disorders of amino acids/fattyacid oxidation defects /organic acidemias, CT-Brain, ophthalmological and ENT evaluation. The cases were then screened for congenital disorder of glycosylation using capillary zone electrophoresis of serum transferrin.

Out of 30 cases three patients had abnormal transferrin pattern i.e. CDT-7.5%, 66.4% , 56.4 %. (The reference interval of CDT is <1.3%) . All three abnormal transferrin patterns observed in our study are consistent with the complete loss of glycans as demonstrated in the studies conducted by Fabienne parente.

This observational study concludes that the prevalence of CDG is about 10% among the 30 cases which is a statistically significant finding. This finding inferes that the prevalence of CDG is abnormally high in the study population. Capillary zone electrophoresis of serum transferrin can be used as a simple technique to screen for this disorder.

The distribution of transferrin isoforms among different age group and sexes were analysed in the control group and it is found that there is no statistical significant difference in the distribution of transferrin isoforms between different age group and sexes. This finding concludes that the distribution of transferrin isoforms are independent of the age and sex. Therefore age and gender specific reference interval for transferrin isoforms may not be important in the diagnosis of CDG.

The distribution of transferrin isoforms among various clinical presentations in the study group was analysed and it is found that statistically significant increase in the aisialotransferrin isoform is present in the group with History of consangineous marriage when compared to the group with nonconsangineous marriage.Since asialo transferrin contributes to the Carbohydrate Deficient Transferrin measure, this finding infers that consangineous marriage is a significant risk factor for congenital disorder of glycosylation.

Similarly the transferrin pattern was compared between cases with positive radiological findings (cerebral atrophy) and cases with absent radiological findings.It is found that statistical significant difference exist in the distribution of asialotransferrin isoforms and CDT between the two groups. This finding infers that presence of radiological features adds as a supportive evidence for the diagnosis of CDG.

Thus this study concludes that:

- From this study we have first reported the presence of congenital disorder of glycosylation in our country.
- The prevalence of congenital disorder of glycosylation is high in study group (10%).
- Capillary zone electrophoresis of serum transferrin can be used as a reliable screening technique to screen this disorder in our population

- Age and gender specific reference interval for transferrin isoforms may not be important in the diagnosis of CDG.
- Consangineous marriage is a significant risk factor for congenital disorder of glycosylation.
- Radiological finding of cerebral atrophy serves as a supportive evidence for the diagnosis of CDG.

Limitations of the study

LIMITATIONS OF THE STUDY

1) Diagnosis of CDG was based upon the reference interval obtained from western studies. No reference interval was established in the study population

2) 2. Accuracy and the precision of the Sebia Capillarys system for the quantification of the transferrin glycoforms was not assessed

3) Capillay zone electrophoresis was not compared and correlated with the gold standard technique of isoelectric focussing of serum transferrin

4) This methodology is only a screening test for CDG . Interferences from hemoglobin, fibrinogen, transferrin polymorphisms, complement fraction C3b & monoclonal immunoglobulins need to be eliminated by using the gold standard IEF technique.

5) O linked glycosylation disorders of CDG cannot be diagnosed using transferrin capillary zone electrophoresis

Future scope of the study

FUTURE SCOPE OF THE STUDY

- Reference interval for CDT glycoforms using capillary zone electrphoresis technique should be established in our population.
- A Panel of tests including capillary zone electrophoresis and other tests to screen CDG should be structred and evaluated to screen this disorder.
- Apo C-III analysis should be included in the panel for diagnosis of O-Linked CDG.

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Annexures

PROFORMA

Name : Age / Sex : Patient ID:

Contact details:

H/O consanguineous marriage of the parents : Pedigree chart:

H/O similar illness in the family :

Significant past medical history :

Any H/O chronic drug intake:

Clinical features	Present	Absent
1.Neurological signs a. psychomotor retardation, b. ataxia, c. hypotonia, d. areflexia, e. seizures		
2. Dysmorphic features a. Facial dysmorphism b. inverted nipples c. cutis laxa		

3.ophthamological signs a. strabismus b. coloboma iris c. retinopathy d. optic atrophy			Investigations done: UMS: TMS: CT Brain Ophthalmological evaluation:
4.Radiological findings a. Cerebral/Cerebellar atrophy, b. Cerebellar hypoplasia			ENT evaluation:
5. Coagulopathy (blood group)			Serum transferrin electrophoresis pattern:
6. protein losing enteropathy			
7. Hypogonadism			

INFORMATION SHEET

Title of the study: "A STUDY ON GLYCOSYLATION PATTERN OF SERUM TRANSFERRIN IN PATIENTS WITH CLINICALLY SUSPECTED CONGENITAL GLYCOSYLATION DISORDERS"

Name	:	Date	:
Age	:	OP No	:
Sex	:	Project Patient No	:

Institution :

- Your venous blood sample of 5ml has been accepted.
- The purpose of this study is to evaluate the use of transferrin electrophoresis in the diagnosis of congenital glycosylation disorder.
- We are selecting certain cases and if your blood sample is found eligible, we may be using your blood sample to perform extra tests and special studies which in any way do not affect your final report or management.
- The privacy of the patients in the research will be maintained throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in this study is voluntary. You are free to decide whether to participate in this study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.
- The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Signature of Investigator

Signature of Participant / Parent

PATIENT CONSENT FORM

Title of the study: "A STUDY ON GLYCOSYLATION PATTERN OF SERUM TRANSFERRIN IN PATIENTS WITH CLINICALLY SUSPECTED CONGENITAL GLYCOSYLATION DISORDERS"

Name	:	Date	:
Age	:	OP No	:
Sex	:	Project Patient No	:
Address	:	Institution:	

- The details of the study have been provided to me in writing and explained to me in my own language.
- I confirm that I have understood the above study and had the opportunity to ask questions.
- I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected.
- I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).
- I have been given an information sheet giving details of the study.
- I fully consent to participate in the above study.

Signature of investigator Signature of participant/parent

சுய ஒப்புதல் கடிதம்

கிளைகோசிலேசன் கோளாருக்கான மருத்துவ அறிகுறிகள் உள்ள நோயாளியின் இரத்தத்தில் டிரான்சுபெரின் எனும் புரதத்தின் கிளைகோசிலேசன் எவ்வாறு உள்ளது என்பதை கண்டறியும் ஆய்வு

பங்கேற்பவரின் பெயர் :

பங்கேற்பவரின் எண்:

- மேலே குறிப்பிட்டுள்ள மருத்துவ ஆய்வின் விவரங்கள் எனக்கு தெளிவாக விளக்கப்பட்டது.என்னுடைய சந்தேகங்களை கேட்கவும், அதற்கான தகுந்த விளக்கங்களை பெறவும் வாய்பளிக்கப்பட்டது.

- நான் இந்த ஆய்வில் தன்னிச்சையாக பங்கேற்கிறேன். எந்த கட்டத்திலும் நான் இந்த ஆய்வில் இருந்து விலகி கொள்ளலாம் என்றும் அறிந்து கொண்டேன்,

- இந்த ஆய்வின் மூலம் கிடைக்கும் தகவல்களையும், பரிசோதனை முடிவுகளையும் மற்றும் சிகிச்சை தொடர்பான தகவல்களையும் மருத்துவர் மேற்கொள்ளும் ஆய்வில் பயன்படுத்தி கொள்ளவும் அதை பிரசுரிக்கவும் என் முழு மனதுடன் சம்மதிக்கிறேன்

- இந்த ஆய்வில் எனக்கு இரத்தப் பரிசோதனை செய்துகொள்ள முழு மனதுடன் சம்மதிக்கிறேன்

- இதன் மூலம் எந்த பின்விளைவும் வராது என மருத்துவர் மூலம் தெரிந்து கொண்டு என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள சம்மதிக்கிறேன் .

பங்கேற்பாளர் கையொப்பம் .....இடம்.....தேதி

கட்டைவிரல் ரேகை

*பங்கேற்பாளர்/*பெற்றோர் *பெயர் மற்றும் விலாசம்*.....
ஆராய்ச்சியாளர் கையொப்பம் .....இடம்.....தேதி

ஆராய்ச்சி தகவல் தாள்

நெருங்கிய இரத்த உறவு திருமணங்களும் அதனால் ஏற்படும் பிறவி கோளாறுகளும் நம் நாட்டில் பரவலாக உள்ளது.கிளைகோசிலேசன் கோளாறு எனும் பிறவி நோயில் நோயாளியின் திசுவிலும் இரத்ததிலும் உள்ள பலவகையான புரதமும், கொழுப்பு அமிலங்களும் கிளைகோசிலேசன் குறைபாடுடன் உள்ளன. இதனால் பலவகையான விவரிக்க முடியாத நரம்பியல் மற்றும் நரம்பியல் அல்லாத பாதிப்புகள் ஏற்படுகின்றது. எனவே இத்தகைய கிளைகோசிலேசன் கோளாருக்கான மருத்துவ அறிகுறிகள் உள்ள நோயாளியின் இரத்தத்தில் டிரான்சுபெரின் எனும் புரதத்தின் கிளைகோசிலேசன் எவ்வாறு உள்ளது என்பதை கண்டறியும் ஆய்வு மேற்கொள்ளப்படுகிறது. இந்த ஆய்விற்கு பிறவி கிளைகோசிலேசன் கோளாறுக்கான மருத்துவ அறிகுறிகளை உள்ள நோயாளியின் இரத்தத்தில் 3 மி.லி இரத்தம் எடுத்து ஆராய்ச்சிக்கு உட்படுத்த உள்ளேன்.

இந்த ஆராய்ச்சியில் உங்களுடைய இரத்தம் சில சிறப்புப் பரிசோதனைகளுக்கு உட்படுத்தப்படும். அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியின் முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது.மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துகொள்கிறோம்.

இந்த சிறப்பு பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லதுஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக்கொள்கிறோம்

ஆராய்ச்சியாளர் கையொப்பம்

பங்கேற்பாளர் /பெற்றோர்
கையொப்பம்

தேதி:

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI 600 003

EC Reg.No.ECR/270/Inst./TN/2013
Telephone No.044 25305301
Fax: 011 25363970

CERTIFICATE OF APPROVAL

To
Dr.A.Preethi
IInd Year P.G. in MD (Bio-Chemistry)
Madras Medical College/RGGGH
Chennai 600 003

Dear Dr.A.Preethi,

The Institutional Ethics Committee has considered your request and approved your study titled " **A STUDY ON GLYCOSYLATION PATTERN OF SERUM TRANSFERRIN IN PATIENTS WITH CLINICALLY SUSPECTED CONGENITAL GLYCOSYLATION DISORDERS" - NO.27012016.**

The following members of Ethics Committee were present in the meeting hold on **05.01.2016** conducted at Madras Medical College, Chennai 3

1.Dr.C.Rajendran, MD.,	:Chairperson
2.Dr.R.Vimala,MD.,Dean,MMC,Ch-3	:Deputy Chairperson
3.Prof.Sudha Seshayyan,MD., Vice Principal,MMC,Ch-3	: Member Secretary
4.Prof.B.Vasanthi,MD.,Inst.of Pharmacology,MMC,Ch-3	: Member
5.Prof.Md.Ali,MD.,DM.,HOD-MGE, MMC,Ch-3	: Member
6.Prof.K.Ramadevi,MD, Director, Inst. of Bio-Chem,MMC,Ch-3	: Member
7.Prof.M.Saraswathi, MD.,Director, Inst.of Path,MMC,Ch-3	: Member
8.Prof.Srinivasagalu,MD.Director,Inst.of Int.Med.MMC,Ch-3	:Member
9.Tmt.J.Rajalakshmi,JAO,MMC,Ch-3	: Lay Person
10.Thiru S.Govindasamy, BA.,BL,High Court,Chennai	: Lawyer
11.Tmt.Arnold Saulina, MA.,MSW.,	:Social Scientist

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

MADRAS MEDICAL COLLEGE
21 JAN 2016
CHENNAI 600 003

Member Secretary – Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
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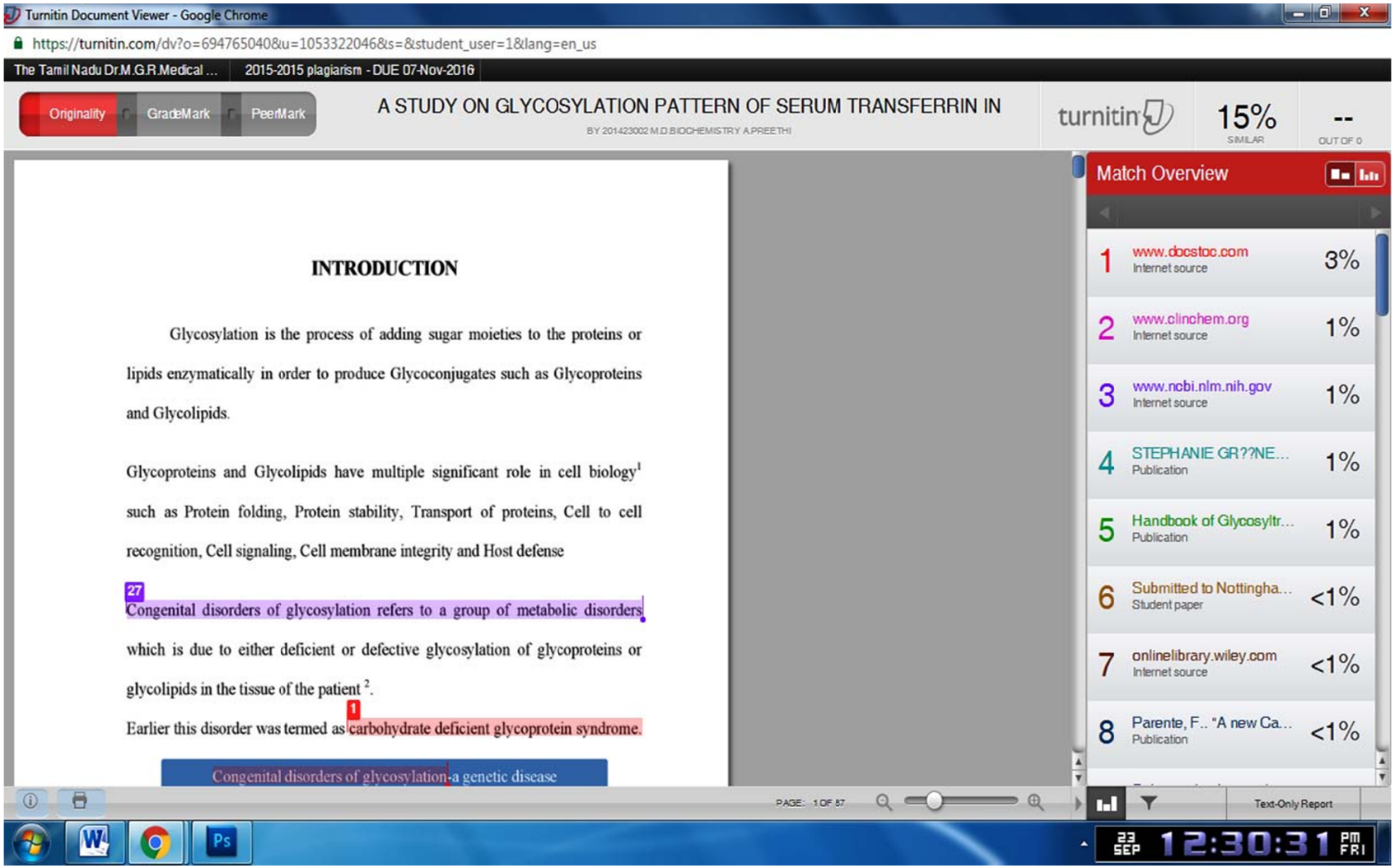
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INTRODUCTION
Glycosylation is the process of adding sugar moieties to the proteins or lipids enzymatically in order to produce Glycoconjugates such as Glycoproteins and Glycolipids.
Glycoproteins and Glycolipids have multiple significant role in cell biology[1] such as Protein folding, Protein stability, Transport of proteins, Cell to cell recognition, Cell signaling, Cell membrane integrity and Host defense
27
Congenital disorders of glycosylation refers to a group of metabolic disorders which is due to either deficient or defective glycosylation of glycoproteins or glycolipids in the tissue of the patient [2].
Earlier this disorder was termed as 1 carbohydrate deficient glycoprotein syndrome.
Congenital disorders of glycosylation-a genetic disease
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